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ENVIRONMENTAL MICROBIOLOGY

AS RELATED TO PLANETARY QUARANTINE

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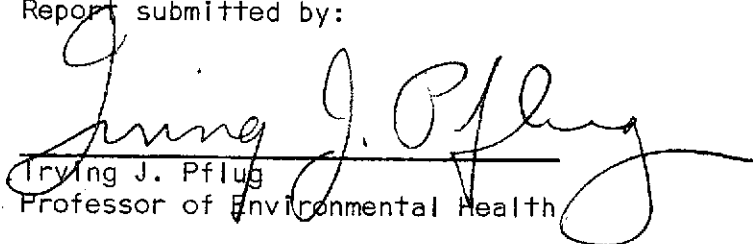Space Science Center at the University of Minnesota

Contributors:

Division of Environmental Health	Division of Biometry
Donald Fisher Geraldine Smith	Jacob Bearman
Bliss Moore	Ronald Jacobson

Report submitted by:

Irving J. Pflug
Professor of Environmental Health

545 Space Science Center
University of Minnesota
Minneapolis, Minnesota 55455

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INTRODUCTION

This report covers research activities during the period December 1, 1971 through May 31, 1972 for the project entitled "Environmental Microbiology as Related to Planetary Quarantine." These studies were conducted by the Division of Environmental Health, School of Public Health, at the University of Minnesota under the auspices of Lawrence B. Hall, Planetary Quarantine Officer, National Aeronautics and Space Administration.

This is the eighth semiannual report of progress on NASA project NGL 24-005-160. A limited number of earlier reports on this project are available for those who do not have a complete set but who wish to obtain all of our procedures and results in a given area.

In this report the section concerned with survival of microbial spores under several temperature and humidity conditions deals totally with the results of experiments conducted using Bacillus subtilis var. niger spores suspended in solutions of sucrose and glycerol at calculated water activities of .85, .90, .99 and heated at temperatures of 45, 60, 75, and 90°C. The overall results of these studies indicate that as the water activity of the liquid decreases from .99 to .85, the heat resistance of the spores increases. It appears that the resistance of the spores is not solely dependent upon the water activity of the environment. The nature of the substance controlling the water activity may also influence the resistance of the spores. In addition, the history of the spores prior to treatment has an effect on their heat resistance at different water activities. The effect of water activity on the heat resistance appears to be less apparent at lower treatment temperatures. In some solutions, the water activity has an effect on the lag time which increases as water activity decreases.

In Appendix A of this report we are including a discussion of the relationships of different indices of water content that are used in dry heat microbial destruction systems. Water is of critical importance in all living systems and in the dry heat destruction area it assumes almost a controlling influence in certain situations. We believe that it is of vital importance for anyone working in this area to understand the fundamental relationships of water in systems and to this end we have assembled this report. We wish to thank the members of the Exotech Corporation who took the time to review the earlier manuscript and work with us to develop this final manuscript. We appreciate

very much their assistance in developing what we believe is a very important report for the NASA Planetary Quarantine area.

We are including as Appendix B a report that was prepared in our laboratory dealing with the design and use of biological indicator systems. To date, biological indicators as such are not being used as part of the Viking system nor are they planned to be used in other missions. However, this is a valuable method for monitoring sterilization processes and it is possible that, in the future, biological indicators may be used to monitor the sterilization cycles for space vehicles.

I. J. Pflug

SURVIVAL OF MICROBIAL SPORES UNDER SEVERAL

TEMPERATURE AND HUMIDITY CONDITIONS

Geraldine M. Smith and Irving J. Pflug

INTRODUCTION

In previous progress reports, we have described investigations concerning the survival of spores suspended in liquids having varying water activities at temperatures below 90°C. These studies were continued and this report presents the complete results of experiments conducted using Bacillus subtilis var. niger spores suspended in solutions of sucrose and glycerol at calculated water activities of .85, .90, and .99 and heated at temperatures of 45, 60, 75, and 90°C.

OBJECTIVE

The objective of these experiments was to determine the survival characteristics of spores in a liquid system at different water activities and at different temperatures.

EXPERIMENTS PERFORMED AND RESULTS OBTAINED

The survival of Bacillus subtilis var. niger spores (Spore Codes AAAA, AADA, and AAOE) suspended in both sucrose and glycerol solutions at calculated water activities (A_w) of .85, .90, and .99 were evaluated at temperatures of 45, 60, 75, and 90°C. The experimental procedures used in performing these tests were described in Progress Report #6. The majority of the tests were done using the AAOE spore crop. These spores were cultured in Synthetic Sporulation Medium 10 (Lazzarini and Santángelo, 1967) and incubated at 32°C. A limited number of tests were conducted using the AAAA and AADA spore crops. Both of these crops were cultured on TAM (Thermoacidurans Agar Modified) agar, supplemented with calcium chloride and magnesium sulfate and incubated at 41°C (Lechowich and Ordal, 1962). All three spore crops were cultured from the same parent strain. Table 1.1 summarizes the tests performed.

TABLE 1.1

Summary of Sucrose and Glycerol Experiments Performed

	Sucrose				Glycerol		
Exp. number	Spore code	temp.	A_w	Exp. number	Spore code	temp.	A_w
GS0211A	AADA	90°C	.99	GS0264A	AAAA	90°C	.99
GS0215A	"	"	.90	GS0265A	"	"	.90
GS0223A	"	"	.85	GS0266A	"	"	.85
GS0293A	AAOE	"	.99	GS0348A	AAOE	"	.99
GS0281A	"	"	.90	GS0349A	"	"	.90
GS0288A	"	"	.85	GS1007A	"	"	.85
GS1341A	"	75°	.99	GS1018A	"	75°C	.99
" "B	"	"	.90	GS1024A	"	"	.90
" "C	"	"	.85	" "B	"	"	.85
GS1067A	"	60°C	.99	GS1137A	"	60°C	.99
" "B	"	"	.90	" "B	"	"	.90
" "C	"	"	.85	" "C	"	"	.85
GS0342A	"	45°C	.99				
" "B	"	"	.90				
" "C	"	"	.85				

The calculated D-values and intercept ratios (IR) are presented in Table 1.2. The data suggest that for spores AADA and AAOE suspended in sucrose the heat resistance (D-value) increased as the water activity decreased. The magnitude of the increase became smaller at the lower heating temperatures. At 90°C the D-value for AAOE increased by a factor of 3.5 as the water activity decreased from .99 to .85 whereas at 45°C the D-value increased only by a factor of 1.5 for the same decrease in water activity.

The AAAA and AAOE spores, which were suspended in glycerol and treated at 90°C, also increased in heat resistance as the water activity decreased. At 75°C the D-value increased only with the change from .99 to .90 A_w. At .85 A_w and .90 A_w the D-values are about the same. At 60°C, no difference in the heat resistance was observed as the water activity decreased.

TABLE 1.2

D-values and Intercept Ratios

Sucrose

Temp. °C	Spore code	.85 A$_w$		.90 A$_w$		.99 A$_w$	
		D-value (hours)	IR	D-value (hours)	IR	D-value (hours)	IR
90	AADA	1.44	.978	.64	.932	.50	.826
90	AAOE	2.39	1.108	1.22	1.096	.69	1.047
75	"	31.91	1.142	19.77	1.123	19.50	.955
60	"	275.92	1.095	179.58	1.123	194.90	.994
45	"	1775.23	1.131	1232.74	1.105	1216.60	1.086
Glycerol							
90	AAAA	.76	.898	.50	.883	.46	.837
90	AAOE	1.44	1.072	.98	1.063	.67	1.049
75	"	27.93	.941	32.37	.816	13.4	1.026
60	"	230.94	1.046	206.94	.993	234.18	1.012

The intercept ratio (log y /log N) is a numerical measure of the shape
of the initial portion of the survivor curve. A value greater than one indi-
cates a lag period in the number of spores killed during the first part of
the heating cycle. A value less than one indicates a greater initial drop in
the number of survivors during the first part of the heating cycle. The
intercept ratios for spores AAOE suspended in sucrose at all heating tempera-
tures are greater than one except at .99 A$_w$ at 60 and 75°C. As the water
activity decreased, the intercept ratio increased indicating a longer lag
period with decreasing water activity. The intercept ratios for glycerol
do not show this phenomenon.

The survivor curves for the AAOE spores which were suspended in sucrose
are presented in Figures 1.1, 1.2, 1.3, and 1.4. At all four temperatures
there is a tendency for the curves to be concave downward, as is indicated
by the intercept ratio. The rate of change in the number of survivors increases
with an increase in heating time. There is a lag period and then the curves
approximate a straight line. As the water activity decreases from .99 to
.90 and to .85, the lag period increases; thus, this effect appears most
pronounced at the lower water activities tested. At the end of the lag phase
the curves tend to be parallel. This suggests that the water activity of the
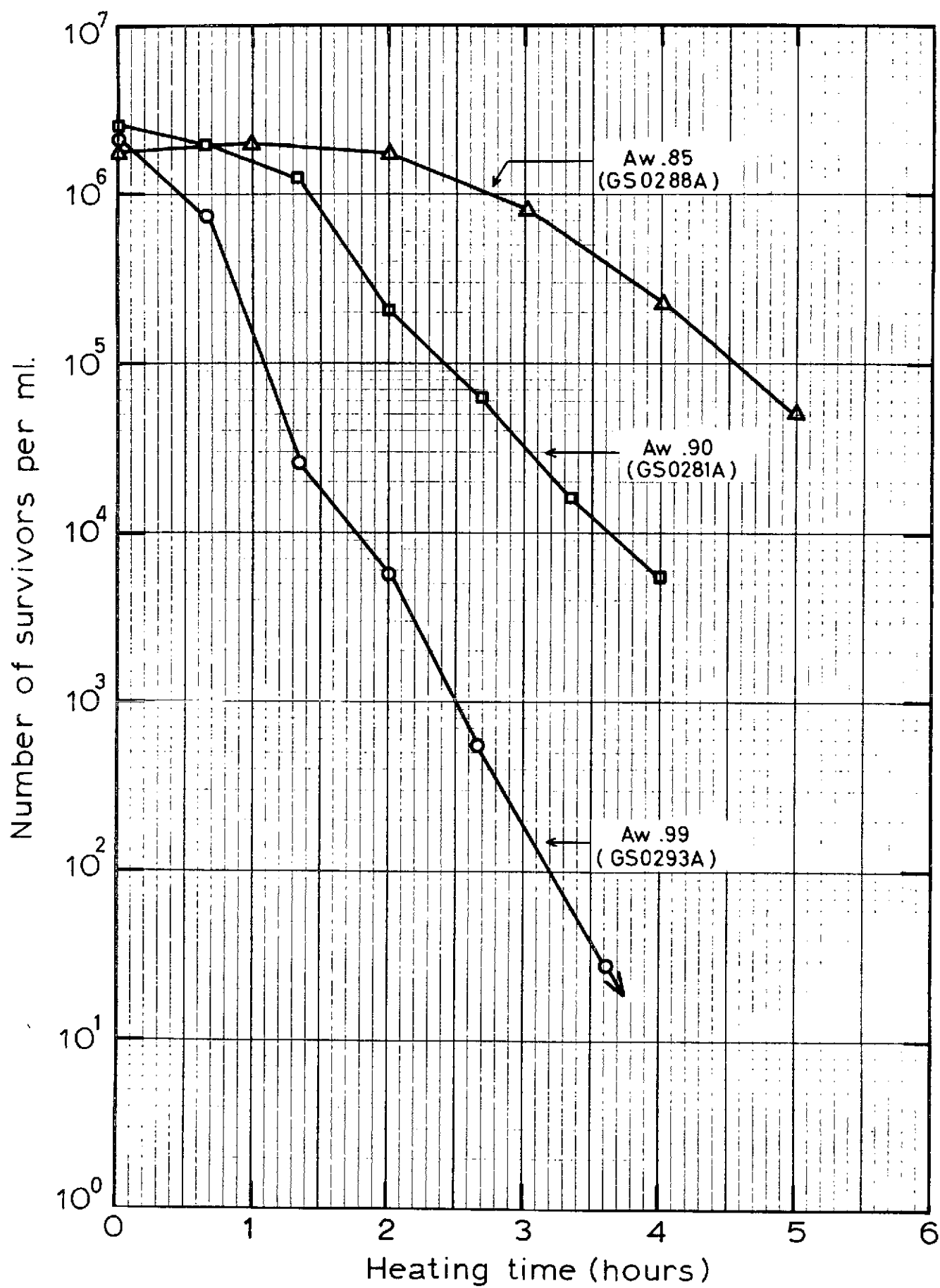sucrose solution has an effect on the lag factor rather than the death rate.

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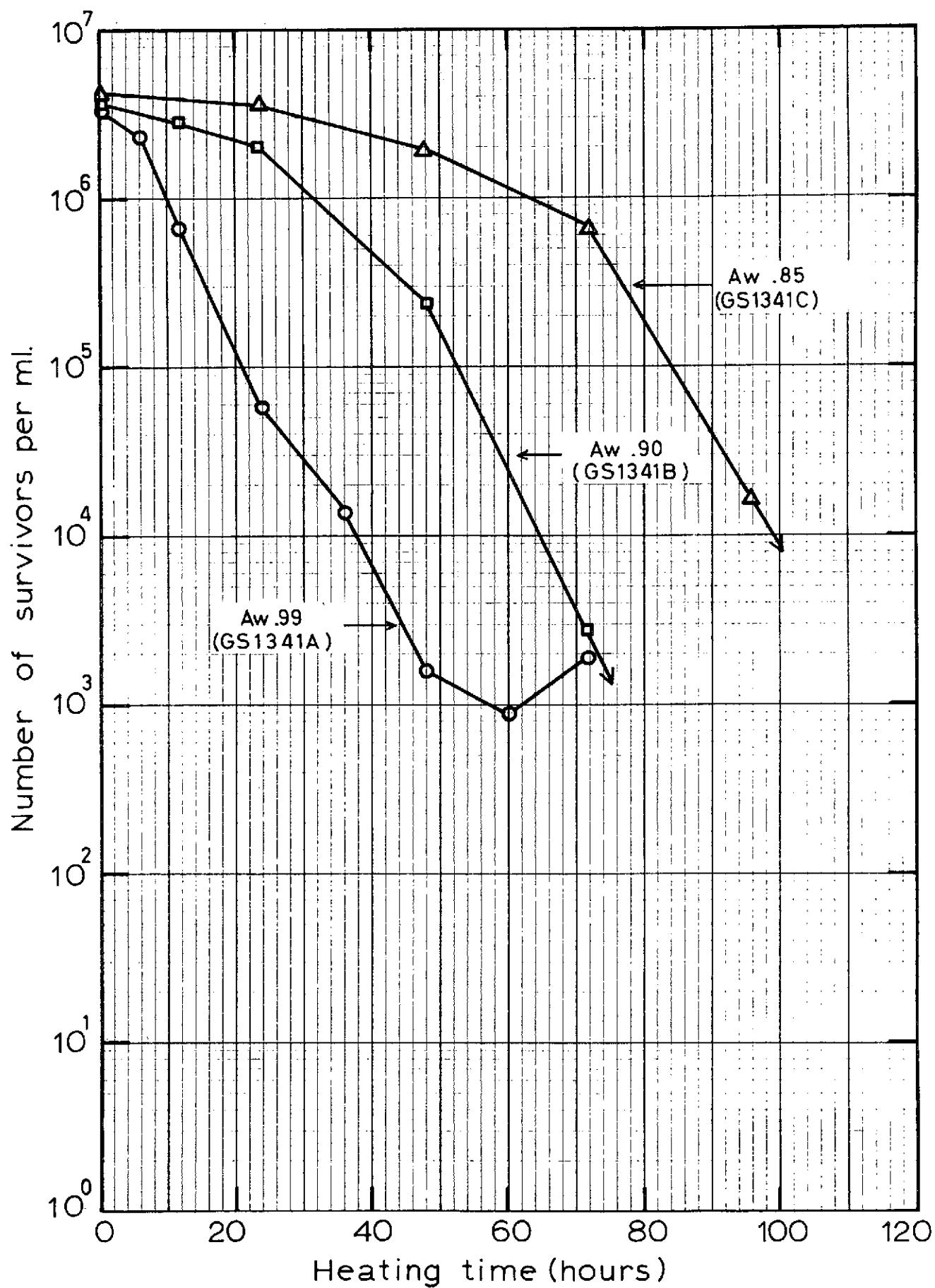Figure I.I: Survivor curves for B. subtilis var. niger spores (AAOE) in sucrose solutions at 90°C.

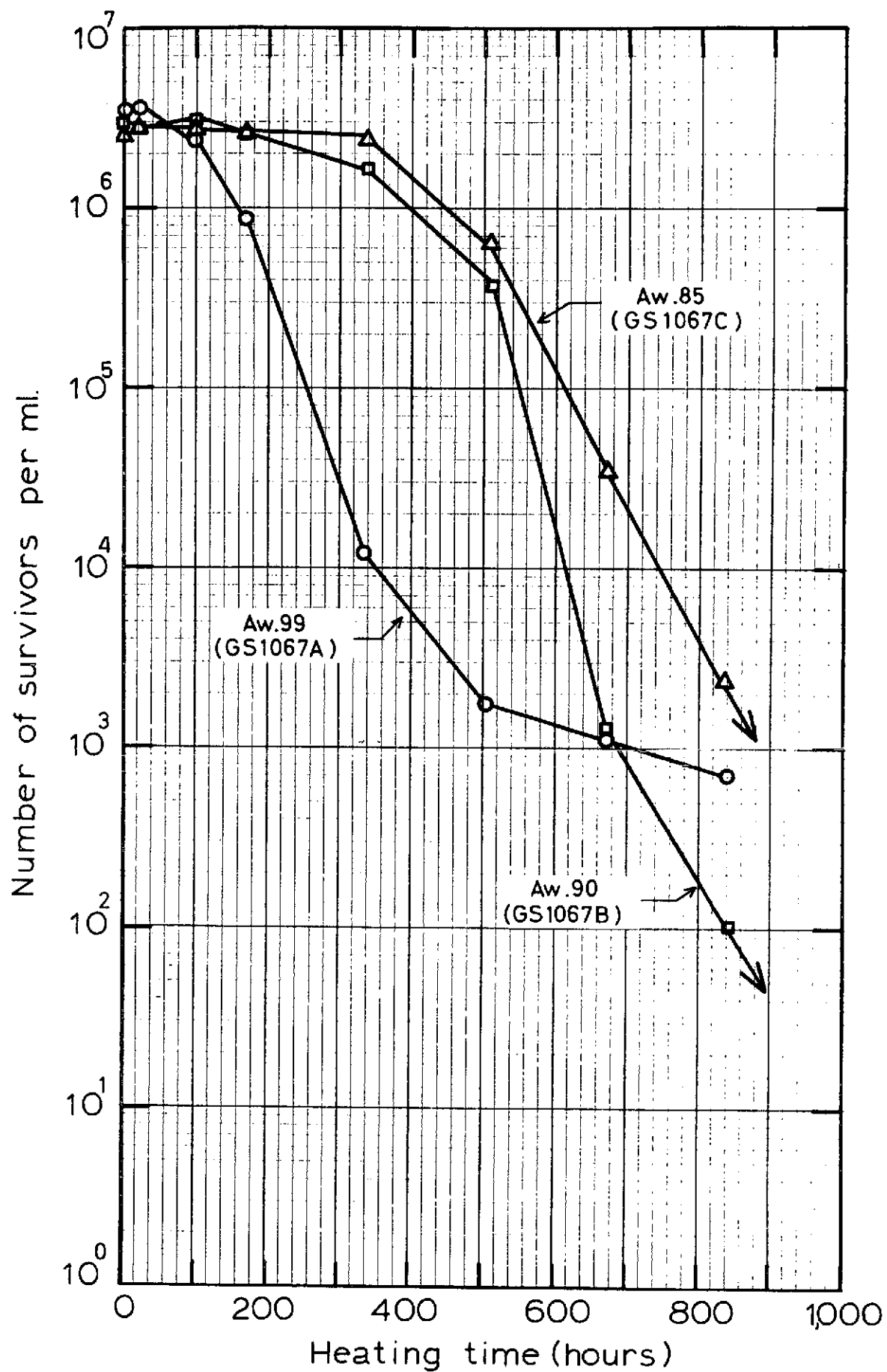Figure I.2: Survivor curves for B. subtilis var. niger spores (AAOE) in sucrose solutions at 75°C.

Figure 1.3: Survivor curves for B. subtilis var. niger spores (AAOE) in sucrose solutions at 60°C.

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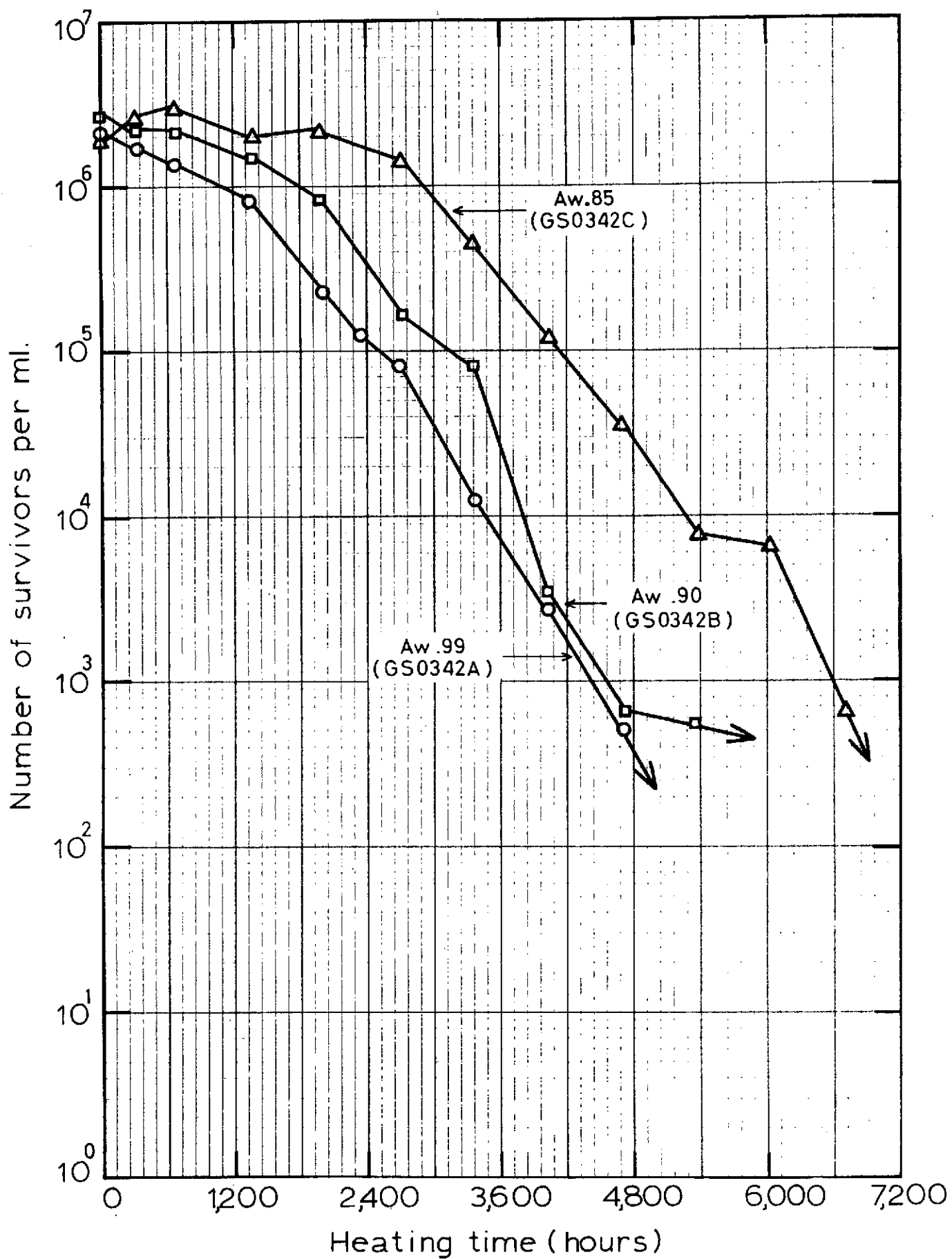Figure I.4: Survivor curves for B. subtilis var. niger spores (AAOE) in sucrose solutions at 45°C.

At 45, 60, and 90°C in .85 A_w sucrose there was actually an increase in the number of colony forming units during the initial part of the treatment cycle. This increase persisted for 2016, 168, and 1 hour respectively at 45, 60, and 90°C. This unexplained increase in the number of spores that were recoverable did not occur with AAOE spores in .85 A_w glycerol or with spores AADA in sucrose at 90°C. The low water activity of the solution and the lack of nutrients rule out the possibility of germination and multiplication. It is possible that a high concentration of dormant spores that would not ordinarily germinate were activated by the sucrose and consequently a larger percentage produced colonies after plating.

Survivor curves for AAOE spores suspended in glycerol are shown in Figures 1.5, 1.6, and 1.7. At 90°C the same initial lag period observed in sucrose is present. However, at 75 and 60°C there is little difference in the curves at the three water activities.

Figures 1.8 and 1.9 show the survivor curves at 90°C for spores AADA in sucrose and spores AAAA in glycerol. These spores also demonstrated increased heat resistance with a decrease in water activity, but a lag period like the one exhibited by the survivor curves for AAOE at 90°C was absent. In all instances the intercept ratio for AADA and AAAA is less than one, whereas the intercept ratio for AAOE in all cases is greater than one. In addition to the difference in the shape of the survivor curves, AAOE is more heat resistant at all water activities than AADA and AAAA.

In Figures 1.10, 1.11, 1.12, 1.13, 1.14, and 1.15, the time in hours for a 1-log (90%), 2-log (99%), and 3-log (99.9%) reduction in spore numbers is plotted as a function of temperature. Table 1.3 presents the z-value in degrees centigrade for spore AAOE in sucrose and glycerol. In sucrose solutions, the temperature change required to produce a 10-fold change in D-value increases as we go to lower temperatures. At 75-90°C a 10-fold change in D-value is brought about with a 10-13° change in temperature (depending upon the water activity) whereas at 45°C an 18° change in temperature is required for a 10-fold change in D-value. For heat treatment at 90°C, the z-value is affected by the water activity of the solution, i.e. the lower the water activity the higher the z-value. In the range of 60-75°C, the z-value is 15° at .99 A_w and 16°C at both .90 A_w and .85 A_w. At 45-60°C the z-value is 18°C at all 3 water activities.
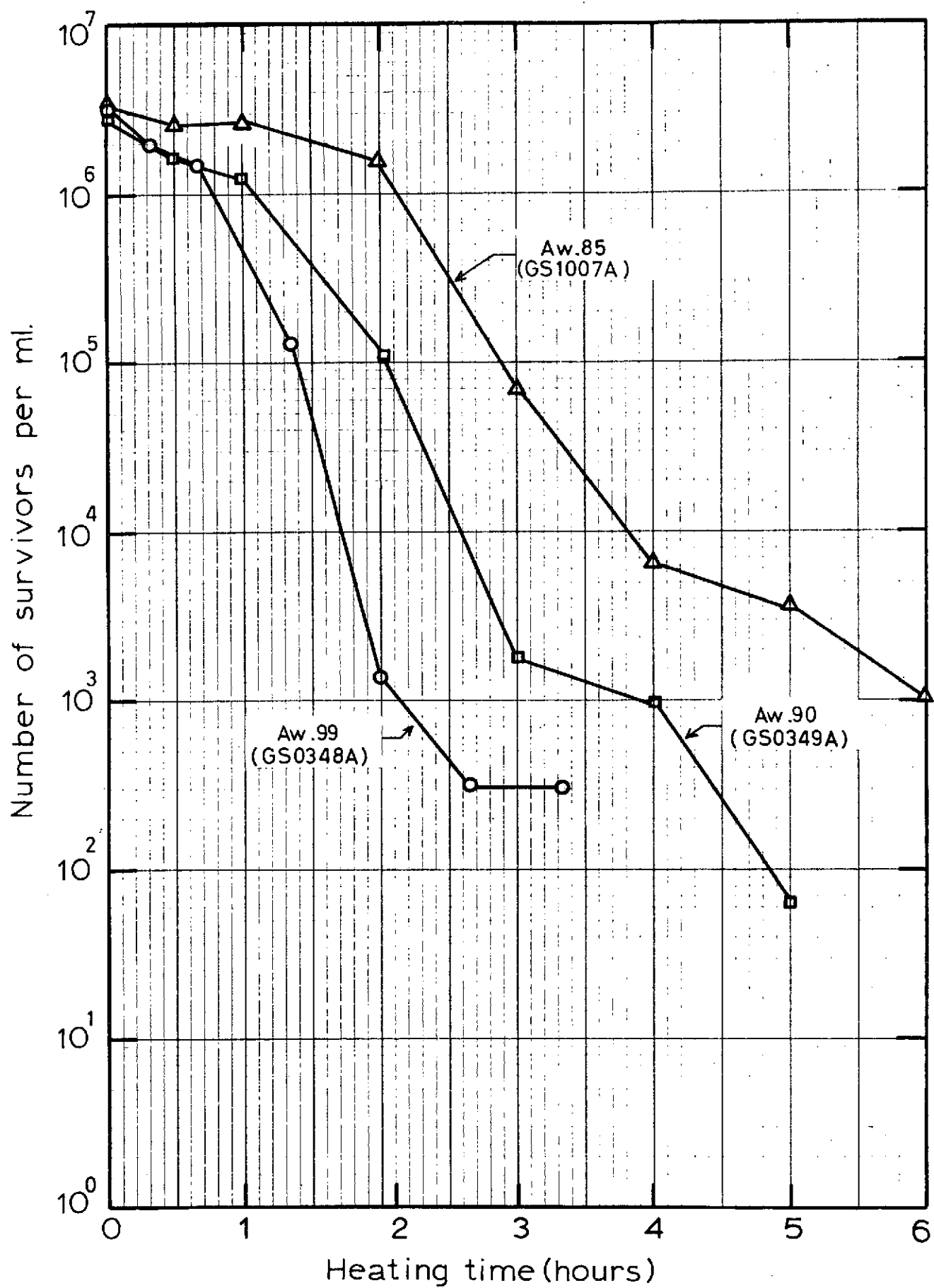
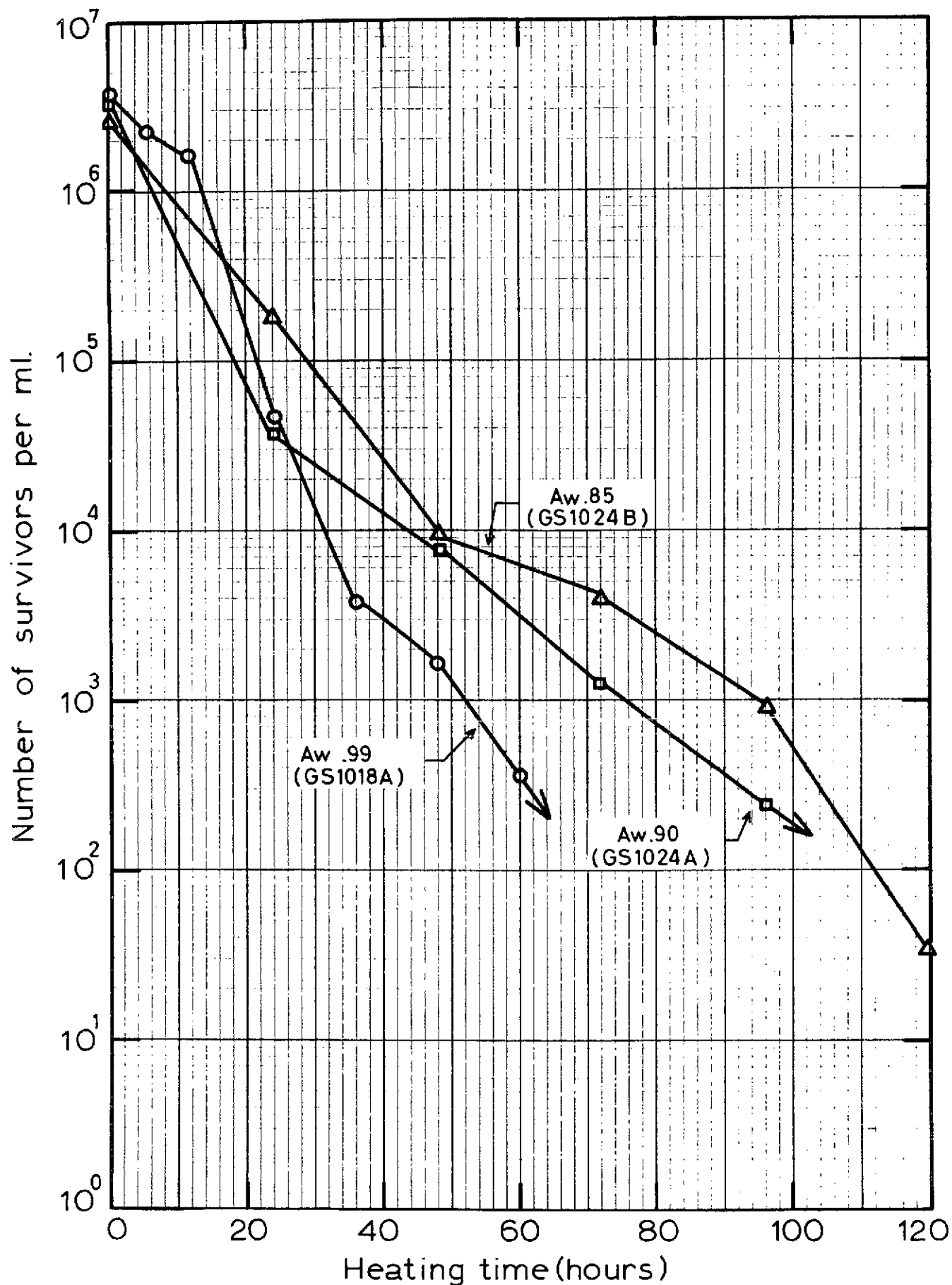Figure 1.5: Survivor curves for B. subtilis var. niger spores (AAOE) in glycerol solutions at 90°C.

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Figure 1.6: Survivor curves for B. subtilis var. niger spores (AAOE) in glycerol solutions at 75°C.

Figure I.7: Survivor curves for B. subtilis var. niger spores (AAOE)
in glycerol solutions at 60°C.
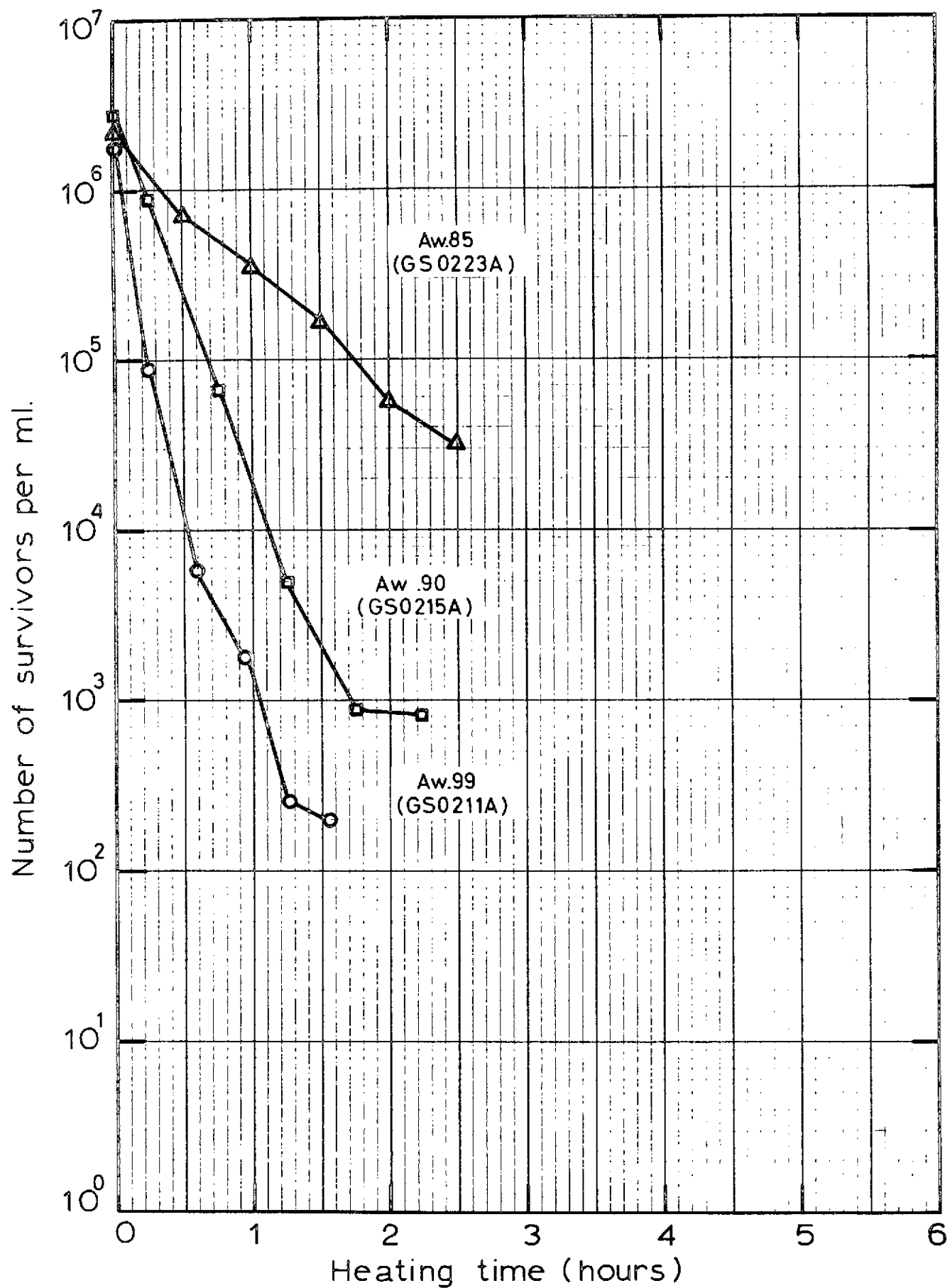
Figure 1.8: Survivor curves for B. subtilis var. niger spores (AADA) in in sucrose solutions at 90°C.
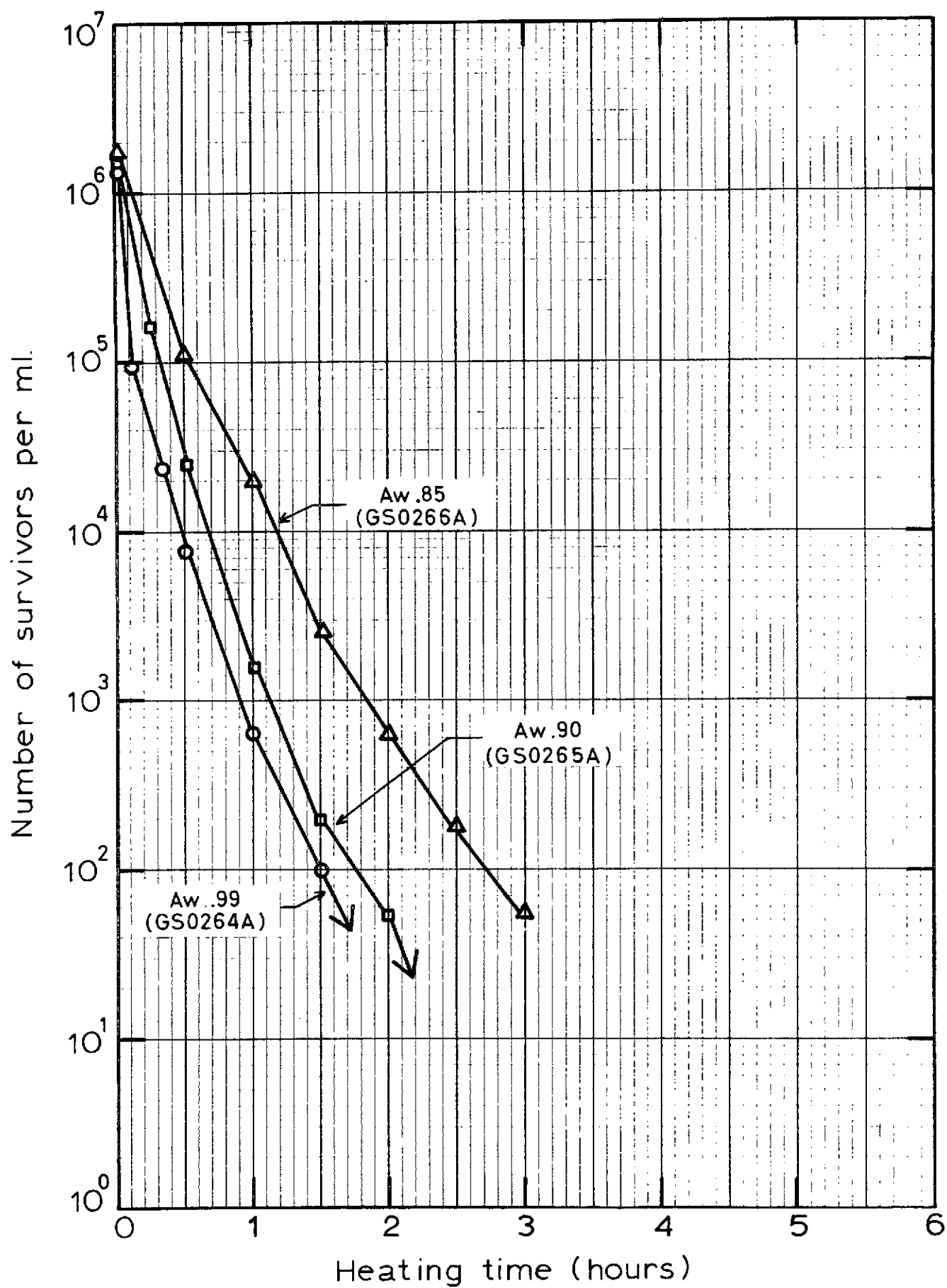
Figure I.9: Survivor curves for <u>B. subtilis</u> var. <u>niger</u> spores (AAAA) in glycerol solutions at 90°C. 13
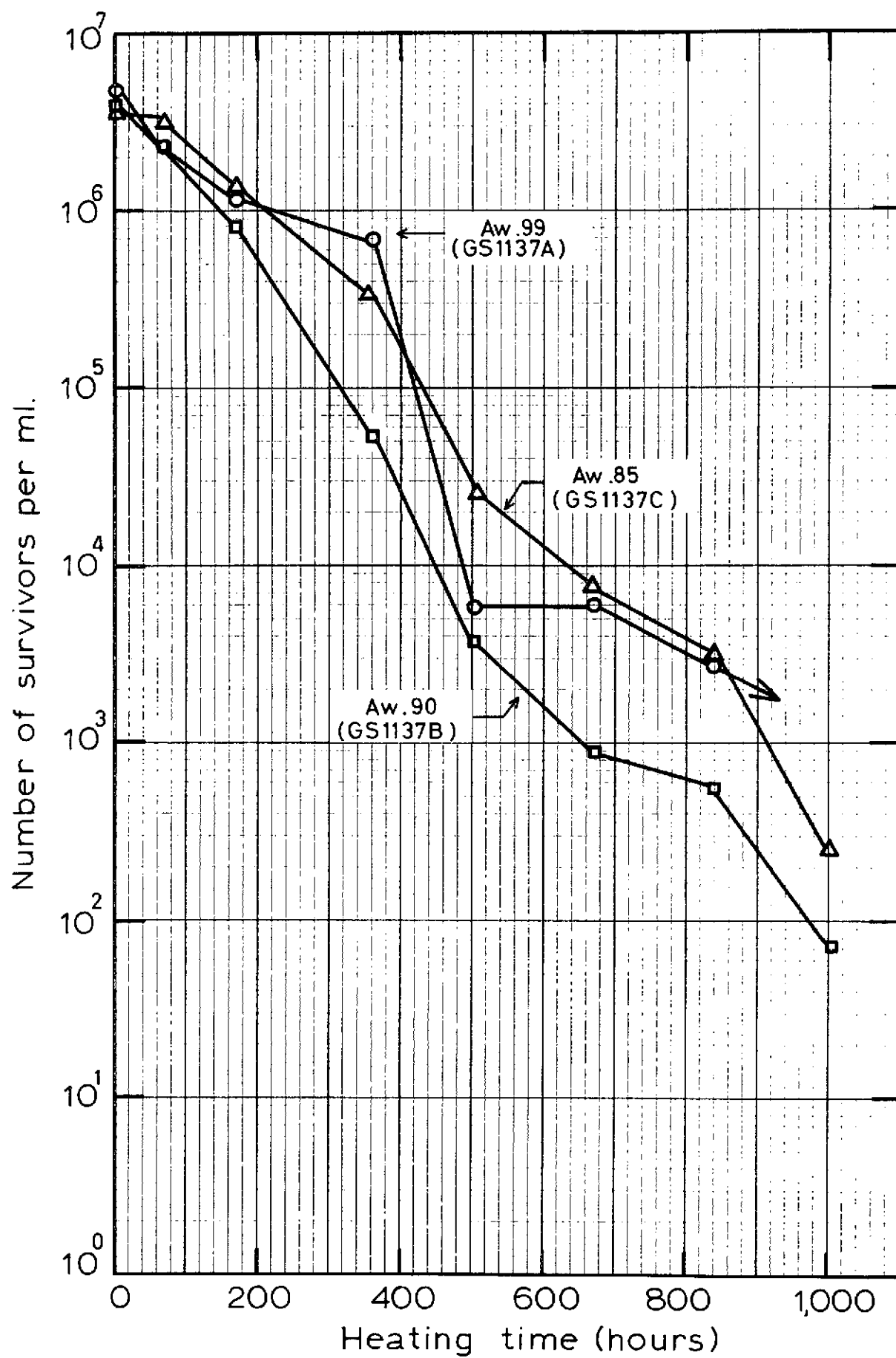
Figure 1.10: Log reduction times vs. temperature for B. subtilis var. niger spores (AAOE) in 0.99 A_w sucrose.

Figure 1.11: Log reduction times vs. temperature for B. subtilis var. niger spores (AAOE) in 0.90 A_w sucrose.

Figure 1.12: Log reduction times vs. temperature for $\underline{B}$. $\underline{subtilis}$ var. $\underline{niger}$ spores (AAOE) in 0.85 A_w sucrose.

Figure 1.13: Log reduction times vs. temperature for B. subtilis var. niger spores (AAOE) in 0.99 A_w glycerol.

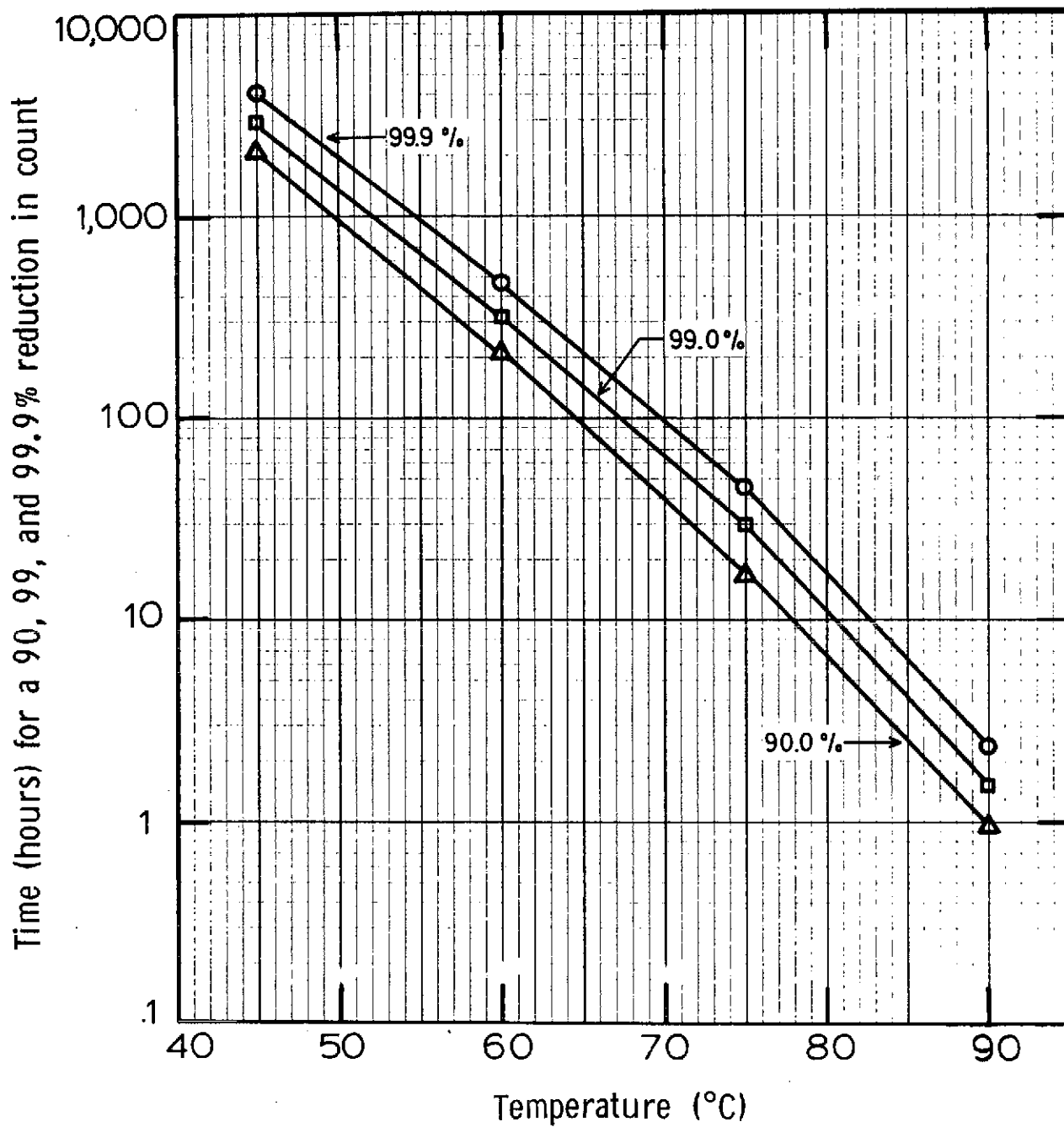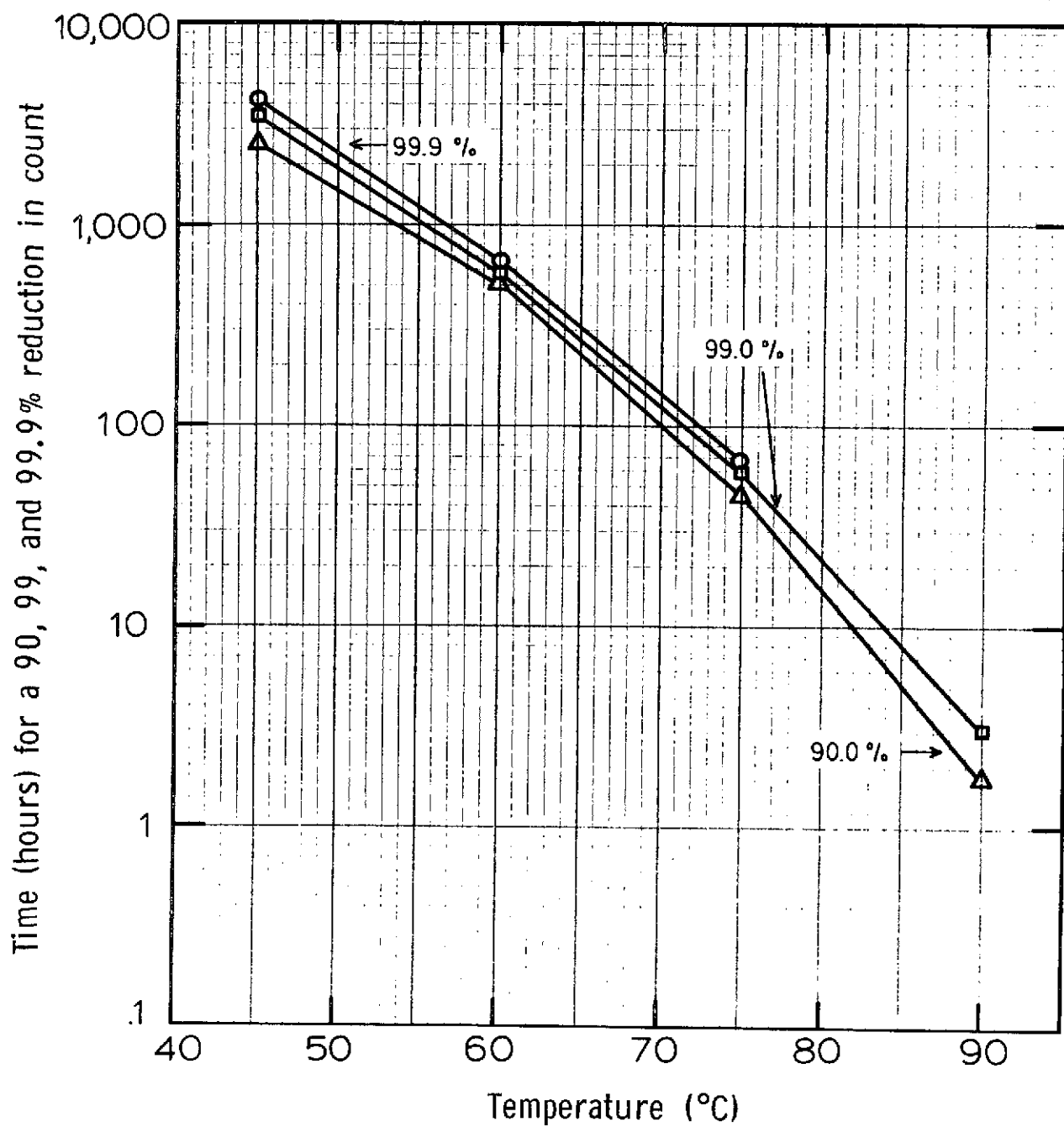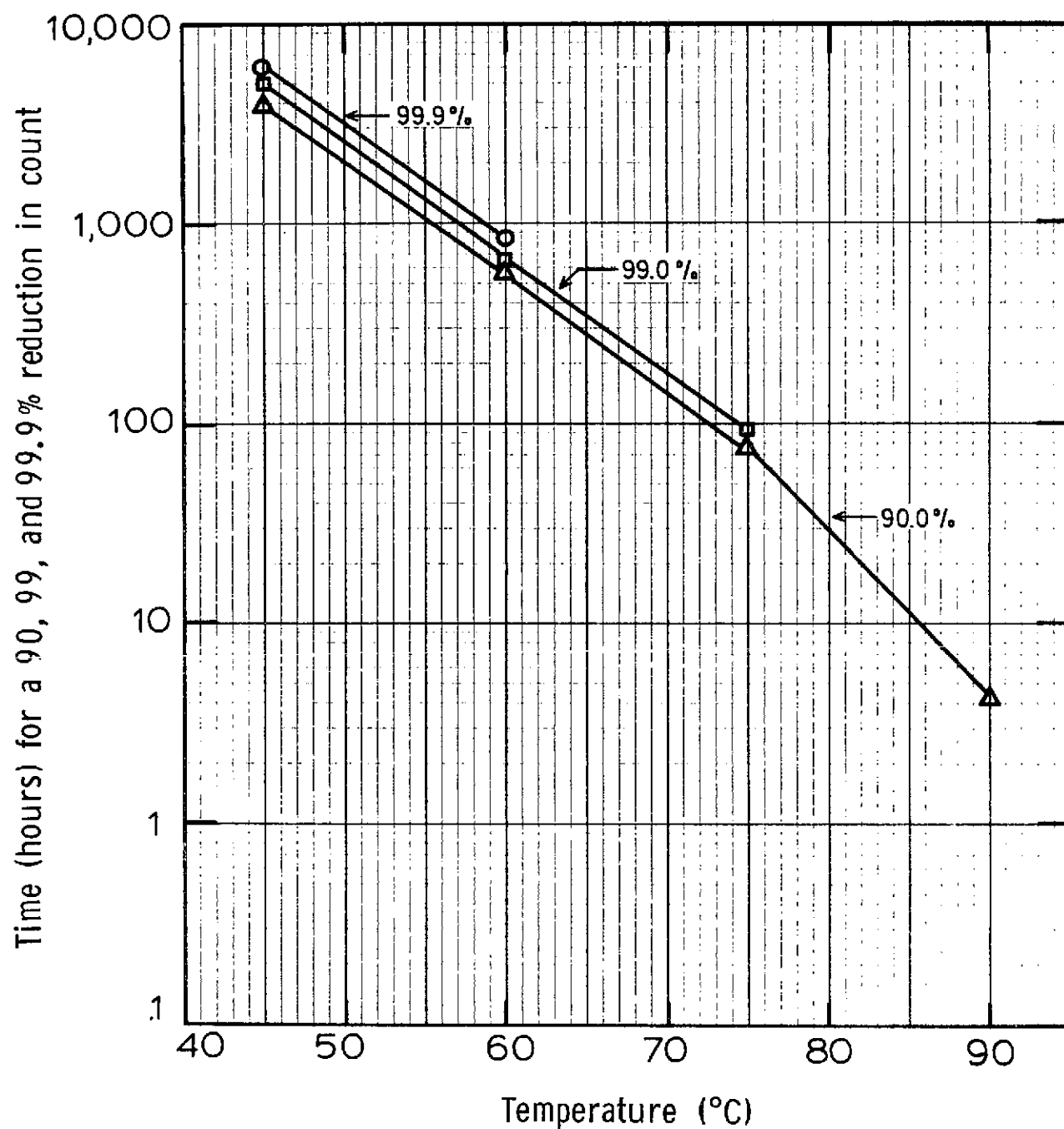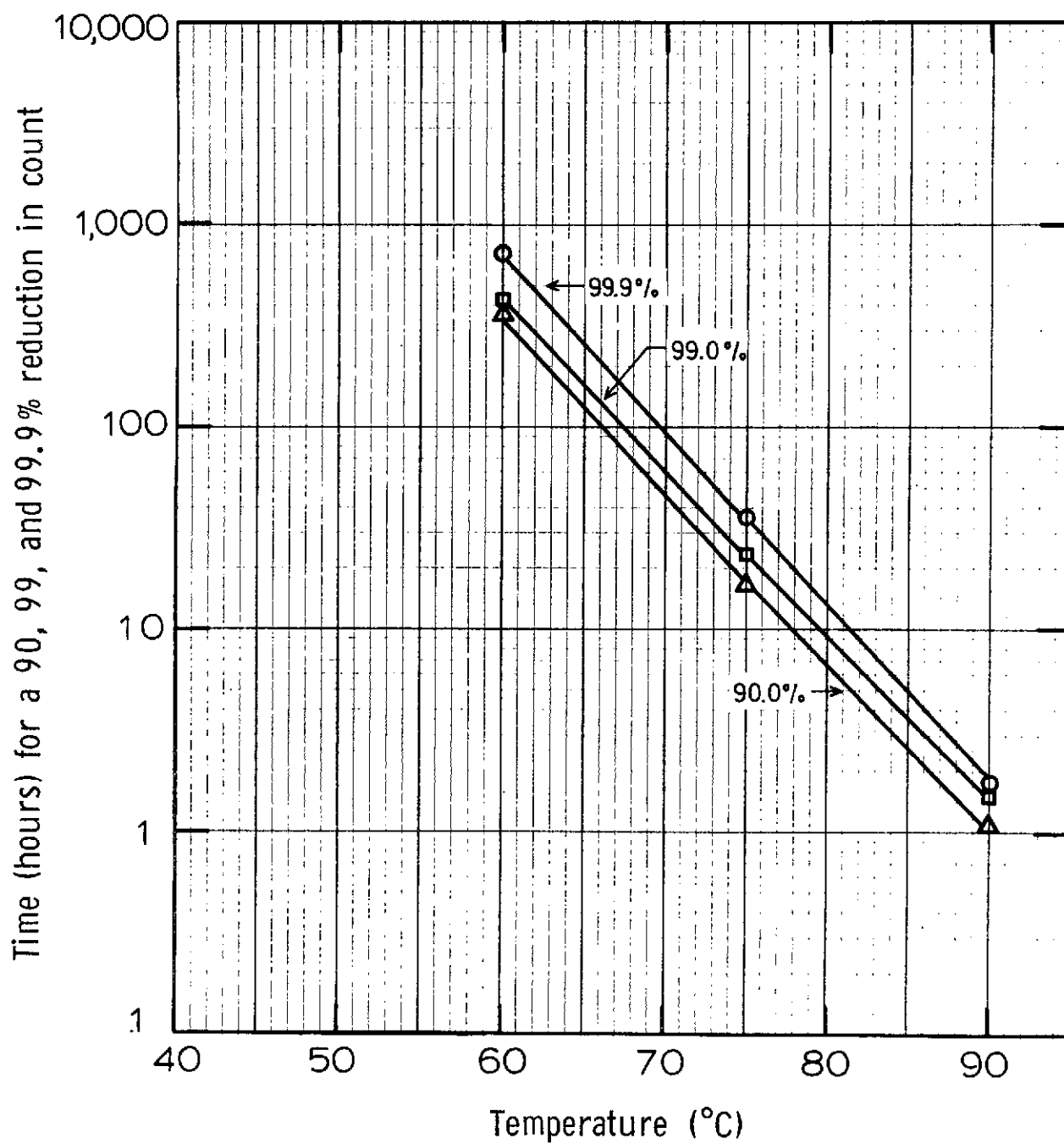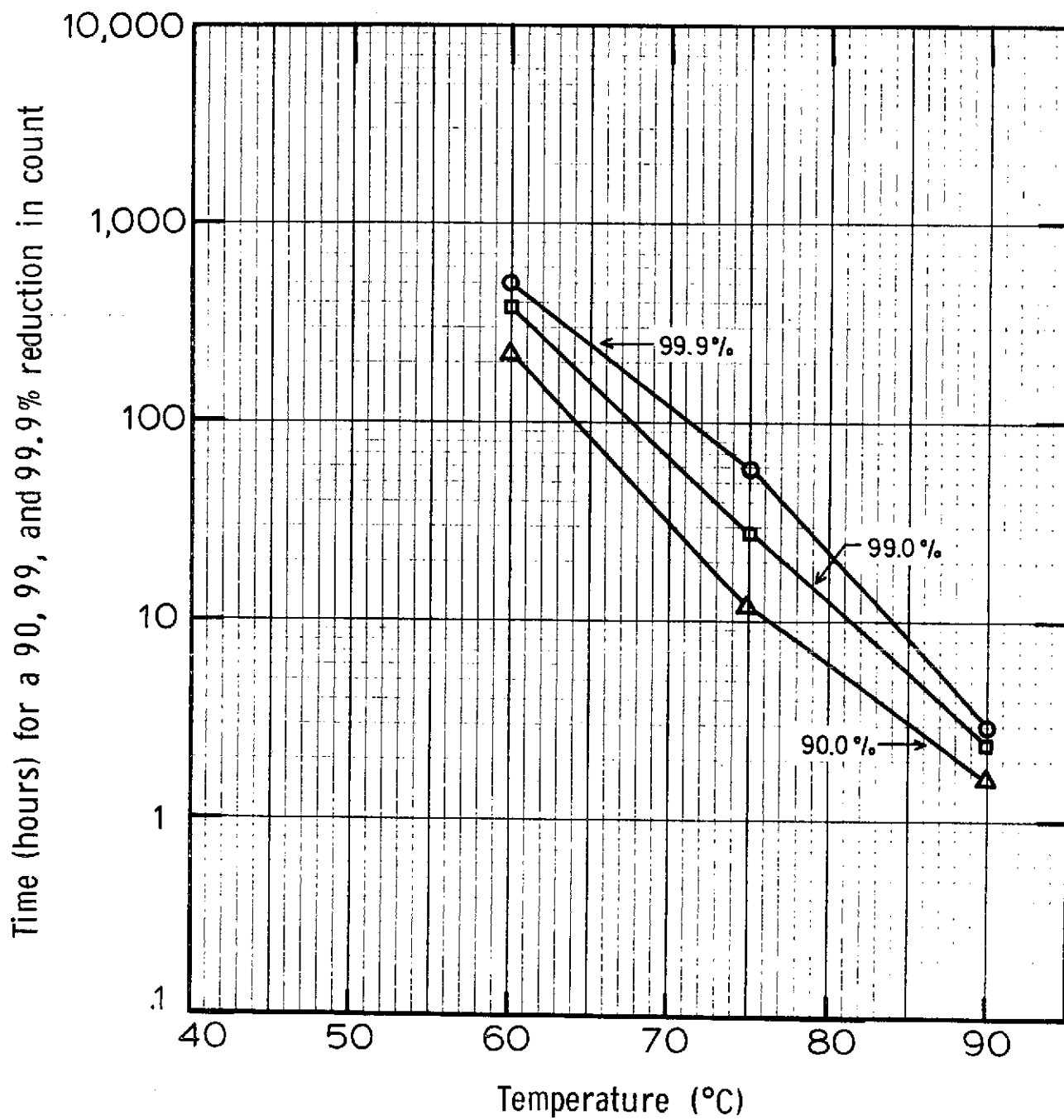Figure 1.14: Log reduction times vs. temperature for B. subtilis var. niger spores (AAOE) in 0.90 A_w glycerol.
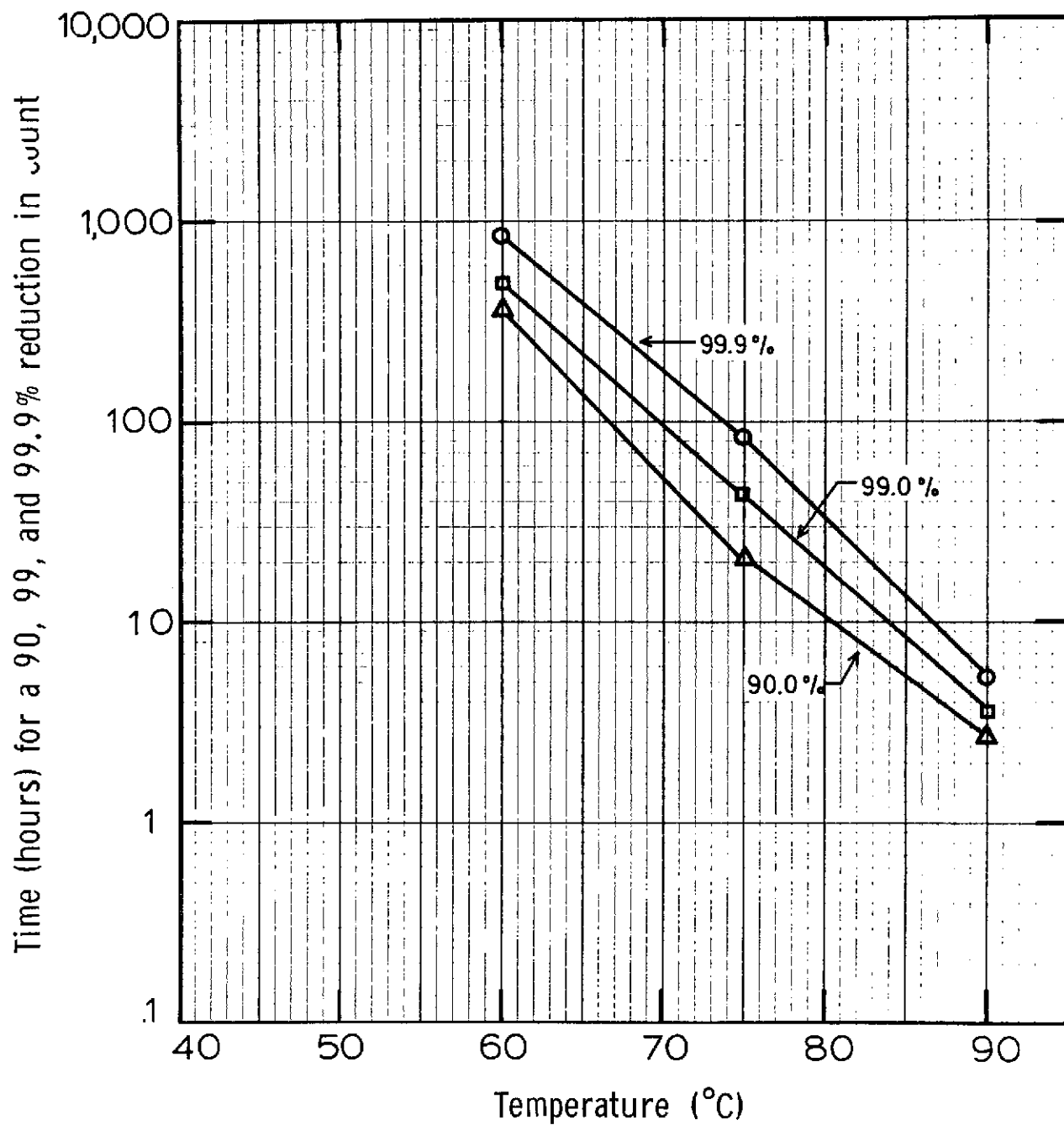
Figure 1.15:   Log reduction times vs. temperature for B. subtilis var. niger spores (AAOE) in 0.85 A_w glycerol.

Table 1.3

Z-values in °C for Spores AAOE
Suspended in Sucrose and Glycerol Solutions

| A_w | Sucrose | | | Glycerol | |
	45-60°C	60-75°C	75-90°C	60-75°C	75-90°C
.99	18°	15°	10°	12°	12°
.90	18°	16°	12°	19°	10°
.85	18°	16°	13°	17°	11°

In the glycerol solutions the increase in z-value as the heating tempera-
ture decreased was not apparent at water activities of .99, but at .90 A_w
and .85 A_w the z-value increased as the temperature decreased.

Figures 1.16 and 1.17 are summary graphs of the time required for a
1-log reduction in survivors for solutions of sucrose and glycerol at all three
water activities. Also included on these graphs are points representing the
length of time required for a 1-log reduction in count for AAOE spores
suspended in buffered distilled water at 90°C and for similar spores deposited
on stainless steel strips treated at 90°C and 1.5% RH.

CONCLUSIONS

The data obtained in these experiments suggest the following conclusions
concerning the survival of spores suspended in solutions of different water
activities.

1. As the water activity of the liquid decreases from .99 to .85, the
heat resistance of the spores increases.

2. The resistance of the spores is not solely dependent upon the water
activity of the environment. The nature of the substance controlling the
water activity may also influence the resistance of the spores.

3. The history of the spores prior to treatment has an affect on their
heat resistance at different water activities.

4. The effect of water activity upon the heat resistance of the spores
is less apparent as the treatment temperature is lowered.

5. In some solutions, the water activity has an effect on the lag time
which increases as water activity decreases. This occurs before the death of
the spores commences.
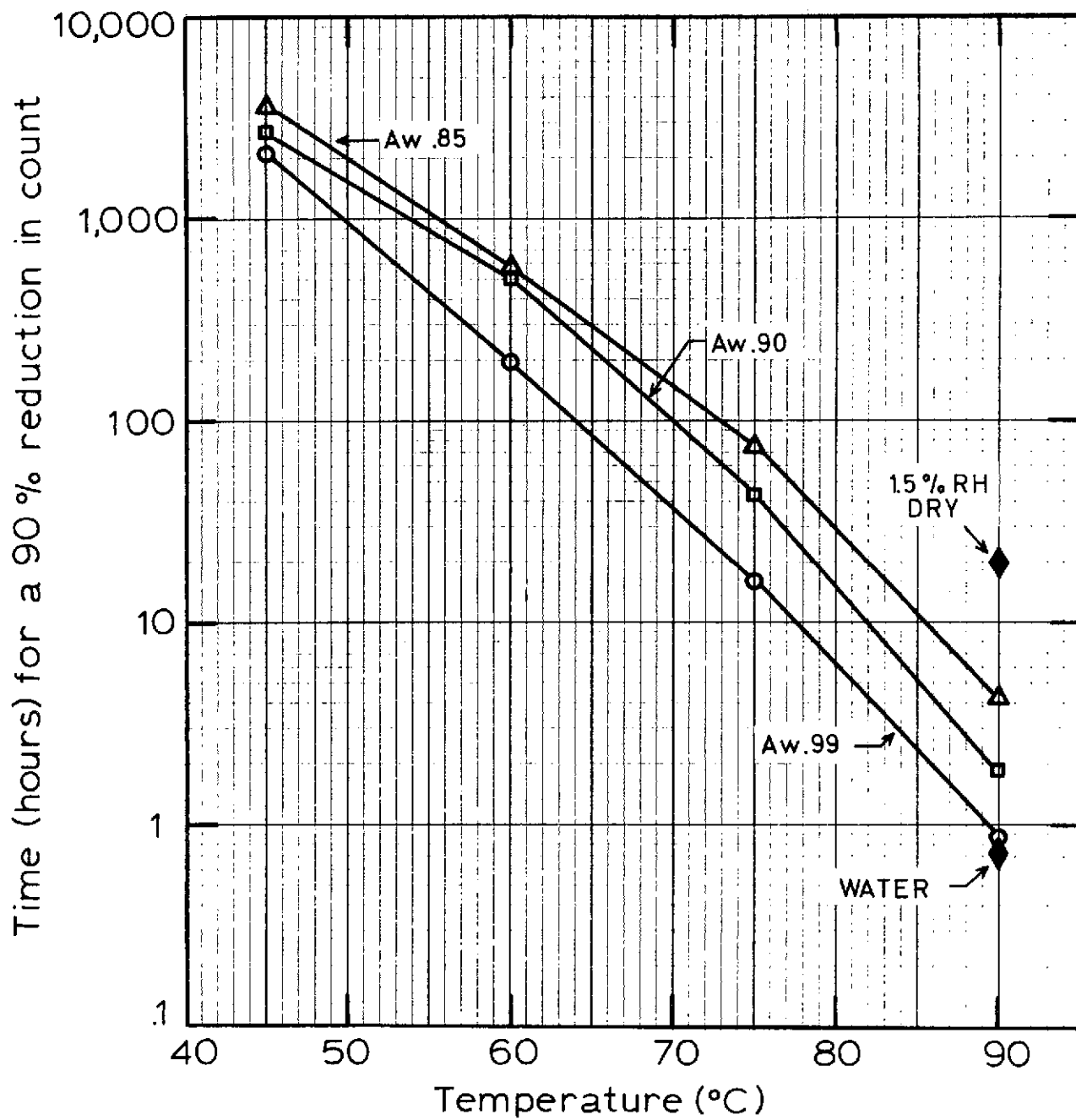
Figure 1.16: Time-temperature plots for one-log reduction of B. subtilis var. niger spores (AAOE) in sucrose solutions with different A_w.

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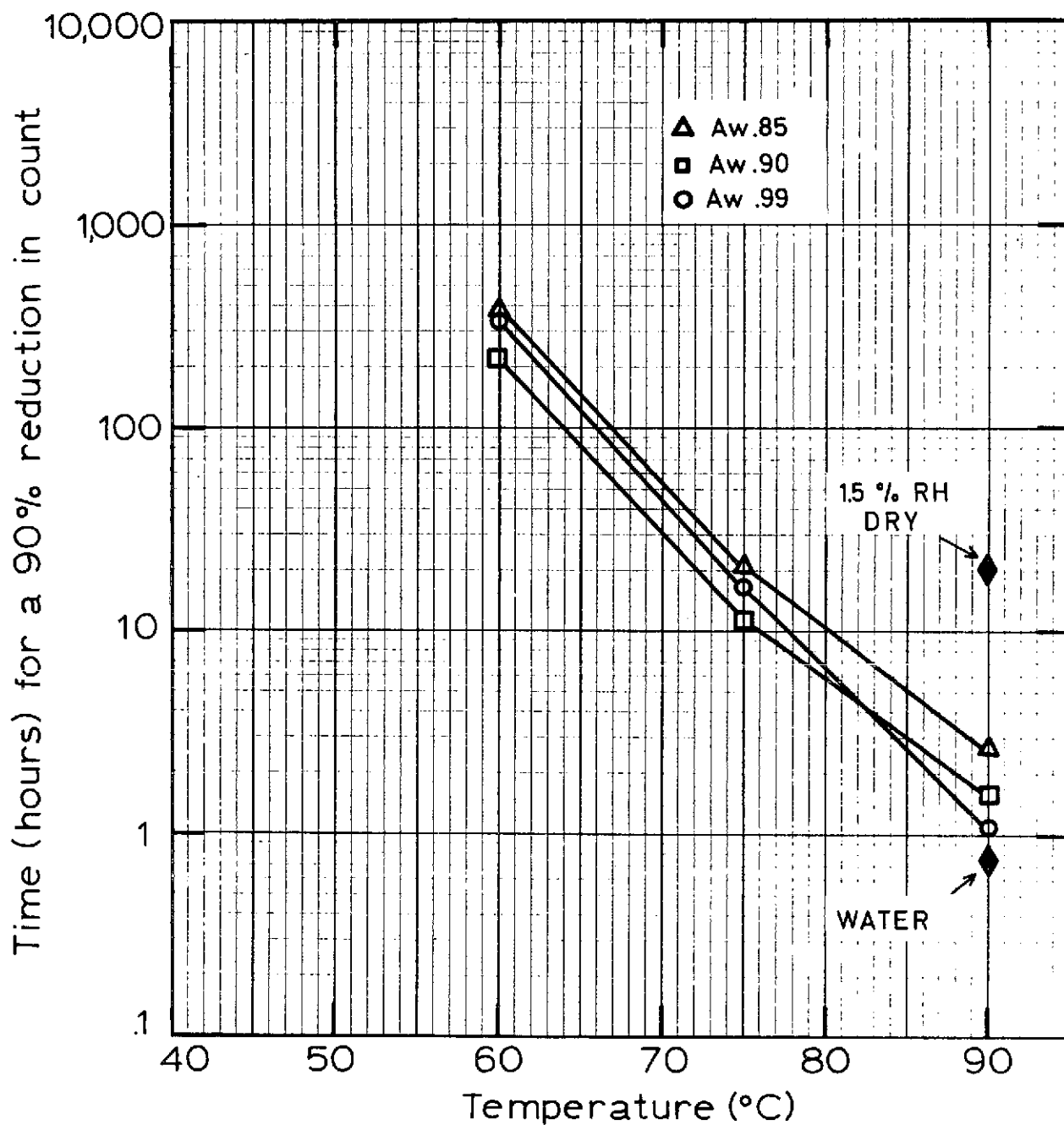
Figure I.17: Time-temperature plots for one-log reduction of B. subtilis var. niger spores (AAOE) in glycerol solutions with different $\overline{A}_w$.

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Lazzarini, R.A.; and E. Santangelo (1967): "Medium-dependent Alteration of Lysine Transfer Ribonucleic Acid in Sporulating Bacillus subtilis Cells," J. of Bacteriol., vol. 94, pp. 125-130.

Lechowich, R.V.; and Z.J. Ordal (1962): "The Influence of the Sporulation Temperature on the Heat Resistance and Chemical Composition of Bacterial Spores," Can. J. Microbiol., vol. 8, pp. 287-295.

PROJECT PERSONNEL

The following personnel all made major contributions to this project: Jacob Bearman, Rebecca Gove, Ronald Jacobson, Irving Pflug, Geraldine Smith, and Yvonne Thun.

DRY HEAT DESTRUCTION RATES OF MICROORGANISMS ON SURFACES

Geraldine M. Smith and Irving J. Pflug

INTRODUCTION

During the past year, the emphasis in our studies of the dry heat destruction characteristics of organisms on surfaces has shifted from using laboratory grown spores to organisms associated with soil. In our previous investigations of the dry heat survival of microorganisms associated with soil particles, ethanol-soil suspensions were used. This work was continued during the current reporting period with our investigation of the dry heat resistance of bacterial spores associated with ethanol-suspended soil at 90°C. We also carried out studies at 90, 110, and 125°C to determine the dry heat resistance of bacterial, mold, and actinomycete spores associated with dry soil particles.

OBJECTIVE

The objective of these studies was to develop a better understanding of the resistance of microorganisms in soil to destruction by dry heat. We were specifically attempting to: (1) compare the heat resistance of organisms in soil suspended in ethanol to organisms in dry soil, (2) compare the heat resistance of mold and actinomycete spores to bacterial spores, and (3) extend the range of treatment temperatures down to 90°C.

MATERIALS AND METHODS

Ethanol-soil Suspension Tests

We carried out one test at 90°C in which we compared ethanol suspensions of Dinkytown soil (XFAIK) with ethanol suspensions of Cape Kennedy soil (WAIIA), particle size < 43 μm. The planchet-boat-hot plate heating system described in Progress Report #7 was used. A test amount of 0.01 ml of soil-ethanol suspension was delivered to each planchet. The hot plate was located in the laminar downflow clean room which was operating at 23°C, 50% RH. The plate count method of assay described in Progress Report #7 was utilized.

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Dry Soil Tests

Dry soil tests were carried out using both Cape Kennedy (WAJJB) and Dinkytown (XFAMA) soil samples. In September 1971, approximately 1,000 g of dry unseived Cape Kennedy soil was received from the United States Public Health Service Laboratory at Phoenix, Arizona. Details of the collection of this soil are given in the Phoenix Quarterly Progress Report #32. The soil was stored in our laboratory at 4°C until March 1972 when it was sieved (in a dry condition). The final sieve size was 43 μm. The Dinkytown soil was collected and sieved in a dry condition in October 1970. The final sieve size was also 43 μm. The Dinkytown soil was stored at room temperature from October 1970 until March 1972.

The soil samples were equilibrated to the atmosphere of the clean room for a period of one week and then suitable portions were weighed into tin-lined thermal death time cups which were 5/16 in. deep x 7/16 in. in diameter. To achieve an N_0 of 1.5–2.0 x 10^6 microorganisms per sample, 0.15 g of Cape Kennedy soil and 0.1 g of Dinkytown soil was placed in each cup. The cups containing the soil were stored in covered glass petri dishes until used.

Copper boats that had been machined to hold 5 cups were used to facilitate the heating of the cups on the hot plate. One boat per time period was used. The number of cups per boat used for each experiment is listed in Table 2.1. The boats holding the cups of soil were placed on the hot plate, and the unit was enclosed by a perforated Lucite cover to protect the samples from the direct flow of air in the clean room. After the prescribed heating time, the boats were removed to a cold plate and cooled for 4 minutes. In some tests, all of the boats for one experiment were not placed on the hot plate on the same day. The placement time schedule was designed to avoid the removal of samples over the weekend.

After cooling, each cup of soil was placed into a screw-cap bottle containing buffered, distilled water. The dry soil particles tended to aggregate into beads approximately .5 ± 1 mm in diameter. Vigorous shaking followed by 5 minutes of insonation dispersed the particles. When a large number of survivors was expected, a second dilution was made by transferring 1 ml of the soil-buffer suspension to a 50 ml dilution blank. Duplicate 0.1 and 1.0 ml aliquots were plated for each cup using each medium. Before every pipetting, the suspension was shaken to prevent settling of the particles

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In test SM2101 only Trypticase Soy Agar pour plates were used. Incubation was at 32°C. After 48 hours of incubation the total number of colonies was counted from the back of the plate without removing the cover. The plates were then reincubated for an additional five days at room temperature. Mold and actinomycete counts were made by observing the macroscopic morphology of the colonies with the aid of a magnifier. The 288, 312, and 336-hour samples were exposed to reduced heat treatment because of intermittent University power failures. Hot plate temperatures were lowered or the unit was off for a total of eight hours over a 48-hour period.

For test SM2126, in addition to the pour plates for total microbial counts with Trypticase Soy Agar, Emerson Agar was used to determine the number of mold and actinomycete colonies. Aliquots of .1 and 1.0 ml were pipetted to the surface of pre-poured Emerson Agar plates and spread with a bent glass rod. The plates were left uncovered until the surface appeared dry. The Trypticase Soy Agar plates were incubated for 48 hours at 32°C and the Emerson Agar plates were incubated for one week at room temperature. Mold and actinomycete colonies were counted and their identification confirmed by microscopic examination. We had some problems with molds spreading over the surface of the Emerson Agar plates. In test SM2173, surface plating was done on both Emerson and Sodium Caseinate Agar. A comparison of the counts showed comparable results on the two media. Spreading was greatly reduced on Sodium Caseinate plates; therefore, the use of Emerson Agar was discontinued.

Table 2.1 summarizes the tests performed using dry soil.

Table 2.1

Summary of Dry Soil Experiments

Test no.	Spore code	Heat. temp.	Total heating time	No. of cups per boat	Type of count	Plating medium
SM2101A	WAJJB	90°C	336 hrs.	4	total	TSA
B	WAJJB	90°C	336 hrs.	4	mold	TSA
C	WAJJB	90°C	336 hrs.	4	actino.	TSA
SM2126A	WAJJB	90°C	1,248 hrs.	2	total	TSA
B	WAJJB	90°C	1,248 hrs.	2	mold	Emer. Agar
C	WAJJB	90°C	1,248 hrs.	2	actino.	Emer. Agar
SM2179A	XFAMA	110°C	336 hrs.	3	total	TSA
B	XFAMA	110°C	336 hrs.	3	mold	Sod. cas.
C	XFAMA	110°C	336 hrs.	3	actino.	Sod. cas.
SM2173A	XFAMA	125°C	56 hrs.	3	total	TSA
B	XFAMA	125°C	56 hrs.	3	mold	Sod. cas.
C	XFAMA	125°C	56 hrs.	3	actino.	Sod. cas.

RESULTS AND DISCUSSION

Figure 2.1 shows the survivor curves at 90°C of spores associated with Cape Kennedy (WA11A) and Dinkytown (XFA1K) soils suspended in ethanol. The general drop in the number of survivors followed by a plateau which was observed in previous work at 125°C is not as apparent at 90°C. If we disregard the N_0 point for the two curves in Figure 2.1, the remainder of the data points for each soil, in general, form a straight line. When the same two soil suspensions were compared at 125°C (Progress Report #7), the plateau portion of the survivor curve for the Cape Kennedy soil occurred at a survivor concentration that was ten times greater than that for the Dinkytown soil. At 90°C, microorganisms in the two soils seem to have the same resistance through 24 hours of heating. After that, the number of survivors in Dinkytown soil drops below the number of survivors in Cape Kennedy soil.
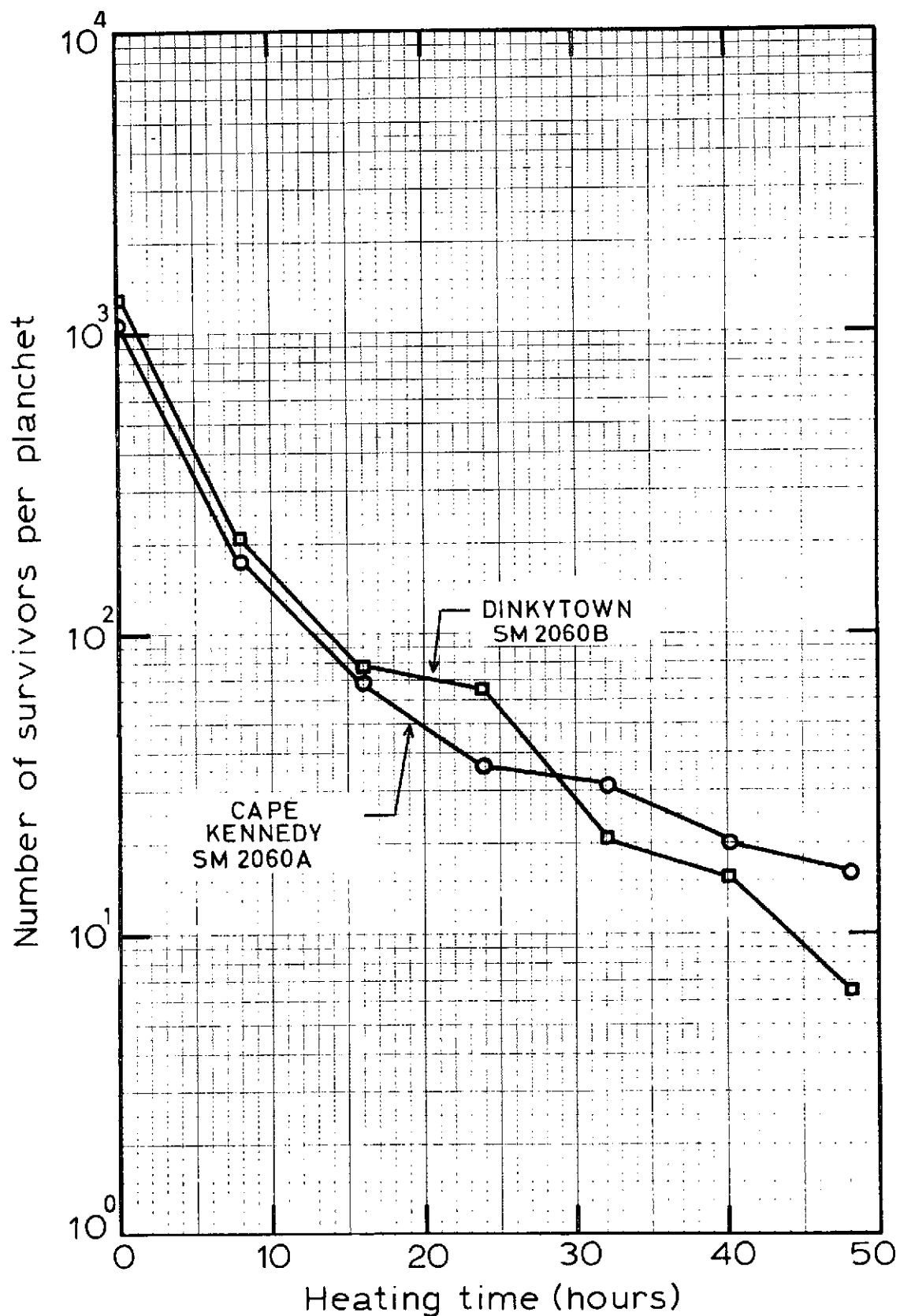
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Figure 2.1: Survivor curves for spores associated with Dinkytown and Cape Kennedy soil suspended in ethanol, heated at 90°C.

We have compared the dry heat resistance of spores associated with
ethanol-suspended soil to spores associated with dry soil. The Dinkytown
soil was tested at 125°C and the Cape Kennedy soil was tested at 90°C. The
results are presented in Figures 2.2 and 2.3. In these graphs the percentage
of survivors is plotted as a function of heating time. The N_0 of the
ethanol-suspended soil was 10^3 organisms per planchet and the N_0 of the
dry soil was 10^6 organisms per cup. The total length of the heating times
at 125°C was eight hours for ethanol-suspended soil and 56 hours for
dry soil. At 90°C the ethanol-suspended soil was heated for a total of
48 hours and the dry soil for 1248 hours. The resistance of microorganisms
associated with soil particles which were not subjected to storage in 95%
ethanol was much greater for both soil samples at each temperature tested.
For Cape Kennedy soil-ethanol suspensions heated at 90°C for 48 hours, the
number of survivors has been reduced by 98.58%; whereas, for the dry Cape
Kennedy soil, 240 hours of heating was required to reduce the number of
survivors by 98.58%. After eight hours of heating at 125°C, the number
of survivors in Dinkytown soil in the ethanol suspension was reduced by
99.9%;whereas, the dry Dinkytown soil required 24 hours of heating at 125°C
to reduce the number of survivors by 99.9%.

The differences observed in these tests could be explained if, (1) the
ethanol dissolved a spore protective substance in the soil-ethanol suspension
or (2) the ethanol caused a reduction in the water content of the spore or
in the water content of the material surrounding the spore thus lowering
its heat resistance.

Table 2.2 shows the number of bacterial, mold, and actinomycete spores
per gram of dry soil (particles size < 43 μm for both soil samples). The
number of the three kinds of organisms in both soils is of the same order of
magnitude. Considering the differences in the extremes of climate, the
proximity to the ocean, the industrialization of the areas, and other
ecological differences in the origin of the samples, the consistency of the
numbers of the classes of microorganisms is rather amazing.

Table 2.2

Comparison of the Number of Microorganisms Per Gram in
Cape Kennedy Soil with the Number in Dinkytown Soil

Soil sample	Total count	Molds	Actinomycetes
Dinkytown	1.97×10^7	8.29×10^5	6.31×10^6
Cape Kennedy	1.25×10^7	5.83×10^5	3.96×10^6

DRY vs. ETOH SUSPENDED DINKYTOWN SOIL, 125°C

Dry, $N_o = 1.94 \times 10^6$
SM 2173 A

ETOH, $N_o = 996$
SM 1292 B

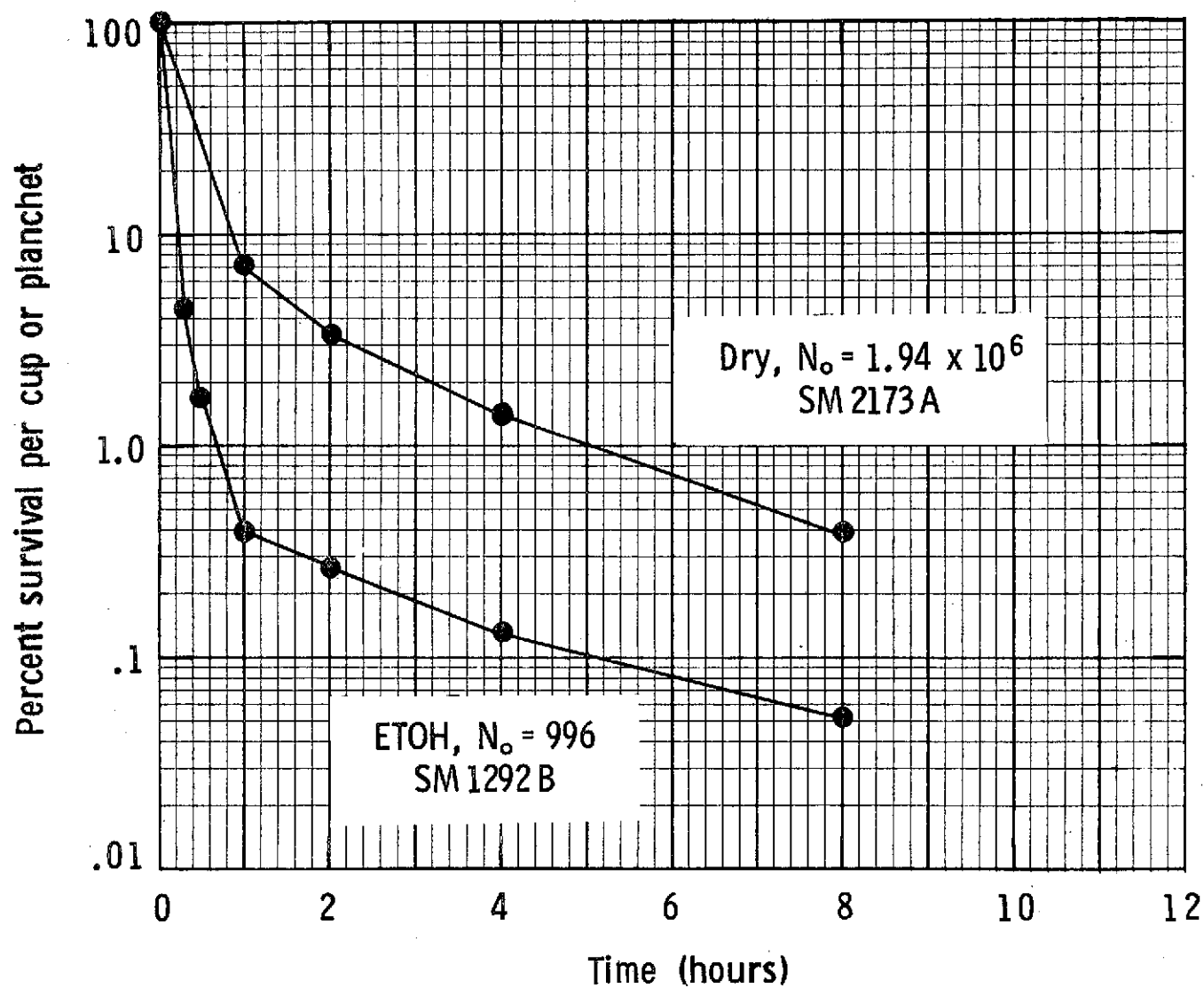Percent survival per cup or planchet

Time (hours)

Figure 2.2: Survival characteristics of organisms in dry Dinkytown soil versus organisms in ethanol suspended Dinkytown soil, heated at 125°C.
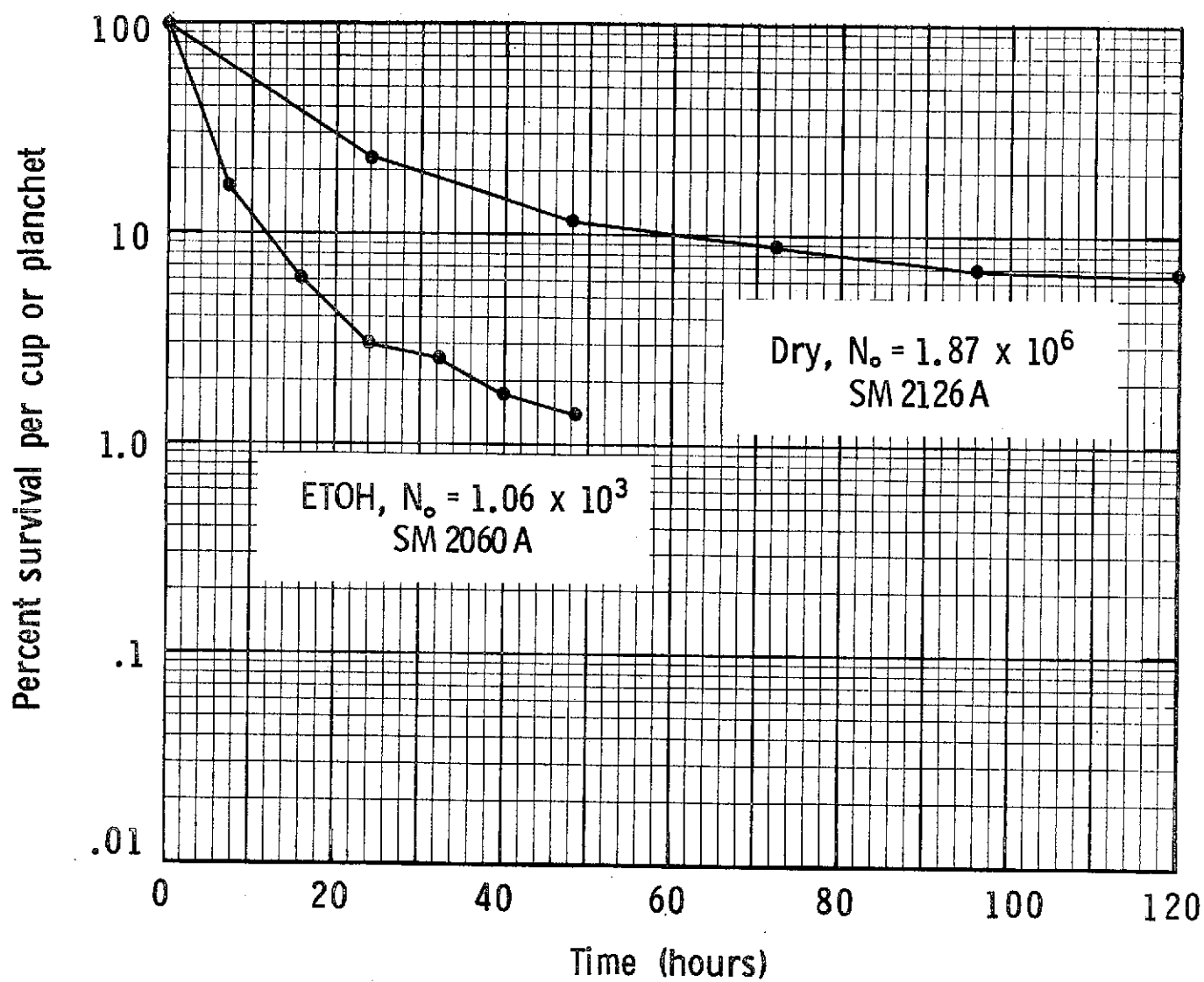
Figure 2.3: Survival characteristics of organisms in dry Cape Kennedy soil versus organisms in ethanol suspended Cape Kennedy soil, heated at 90°C.

In Figures 2.4, 2.5, 2.6, and 2.7 the survivor curves for bacterial, mold, and actinomycete spores are presented at temperatures of 90, 110, and 125°C.

Two tests, SM2101 and SM2126, were conducted at 90°C using dry Cape Kennedy soil. In tests SM2101A, B, and C, samples were taken at 24-hour intervals for a total heating time of 336 hours. All three types of micro-organisms survived 336 hours of treatment. During the latter portion of the heating cycle in tests SM2101A, B, and C, the power was off intermittently for a total of eight hours during a 48-hour period. This power interruption may account for the survival of molds at 336 hours.

In tests SM2126A, B, and C, samples were taken every three to four days for a total heating time of 1248 hours. After 240 hours of heating, no molds were observed. After 1248 hours of heating at 90°C, the bacterial spore count was reduced by 3 logs and the actinomycete spore count was reduced by 4 logs.

Tests at 125°C (SM2173) and at 110°C (SM2179) were carried out using dry Dinkytown soil. In tests SM2173A, B, and C, at 125°C, samples were taken after 1, 2, 4, 6, 16, 24, 32, 48, and 56 hours of heating. The molds survived four hours of heating. After 56 hours of heating at 125°C, the number of surviving bacterial spores was reduced by 3.5 logs and the number of actinomycete spores was reduced by 4 logs. In tests SM2179A, B, and C, samples were analyzed every 24 hours for a total of 336 hours of treatment at 110°C. No molds were detected after 48 hours. After 336 hours of heating, the number of surviving bacterial spores had been reduced by almost 4 logs and the number of surviving actinomycete spores had been reduced by 5 logs.

CONCLUSIONS

1. The shapes of the survivor curves for spores associated with both Dinky-town and Cape Kennedy soil heated at 90°C are similar. The initial rapid die-off and subsequent plateau observed at 125°C is absent.

2. Spores associated with soil particles are more resistant to dry heat when the soil sample has been handled dry than when the soil was suspended in 95% ethanol.

3. Bacterial spores are the most dry heat resistant organisms associated with soil particles. Actinomycete spores are less resistant than bacterial spores and mold spores are the least resistant.
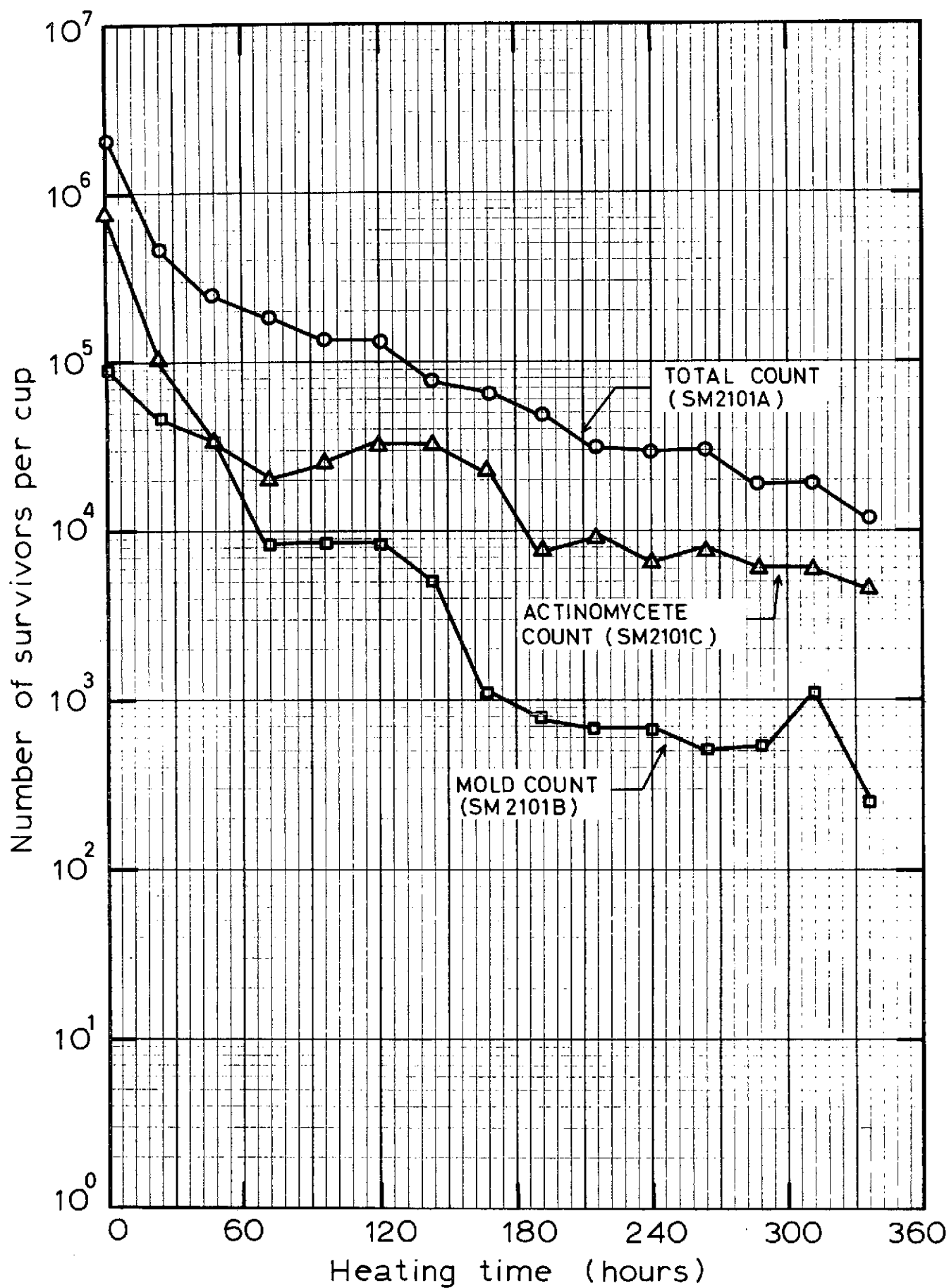
Figure 2.4: Survival characteristics of bacterial, mold, and actinomycete spores associated with dry Cape Kennedy soil, heated at 90°C.
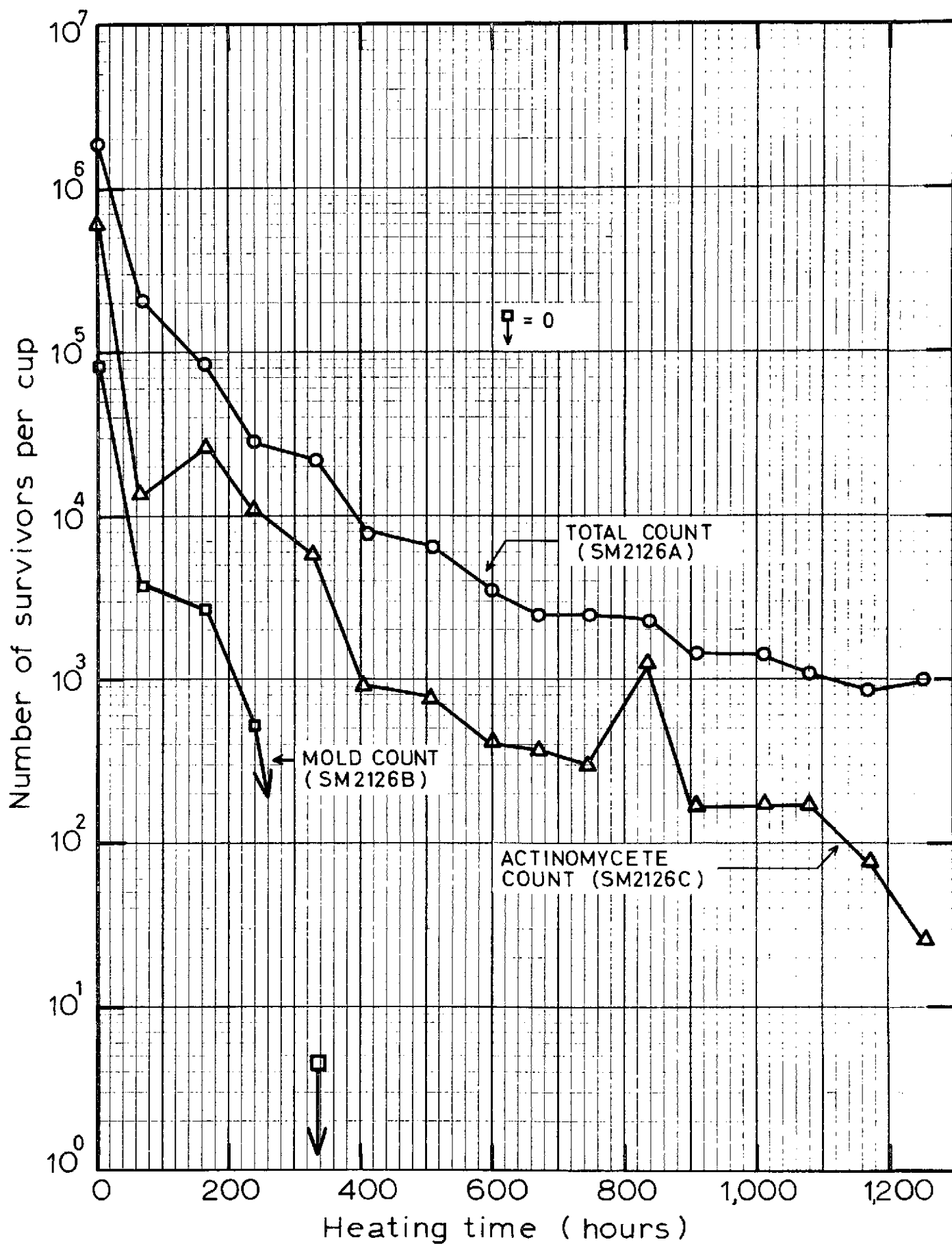
Figure 2.5: Survival characteristics of bacterial, mold, and actinomycete spores associated with dry Cape Kennedy soil, heated at 90°C.
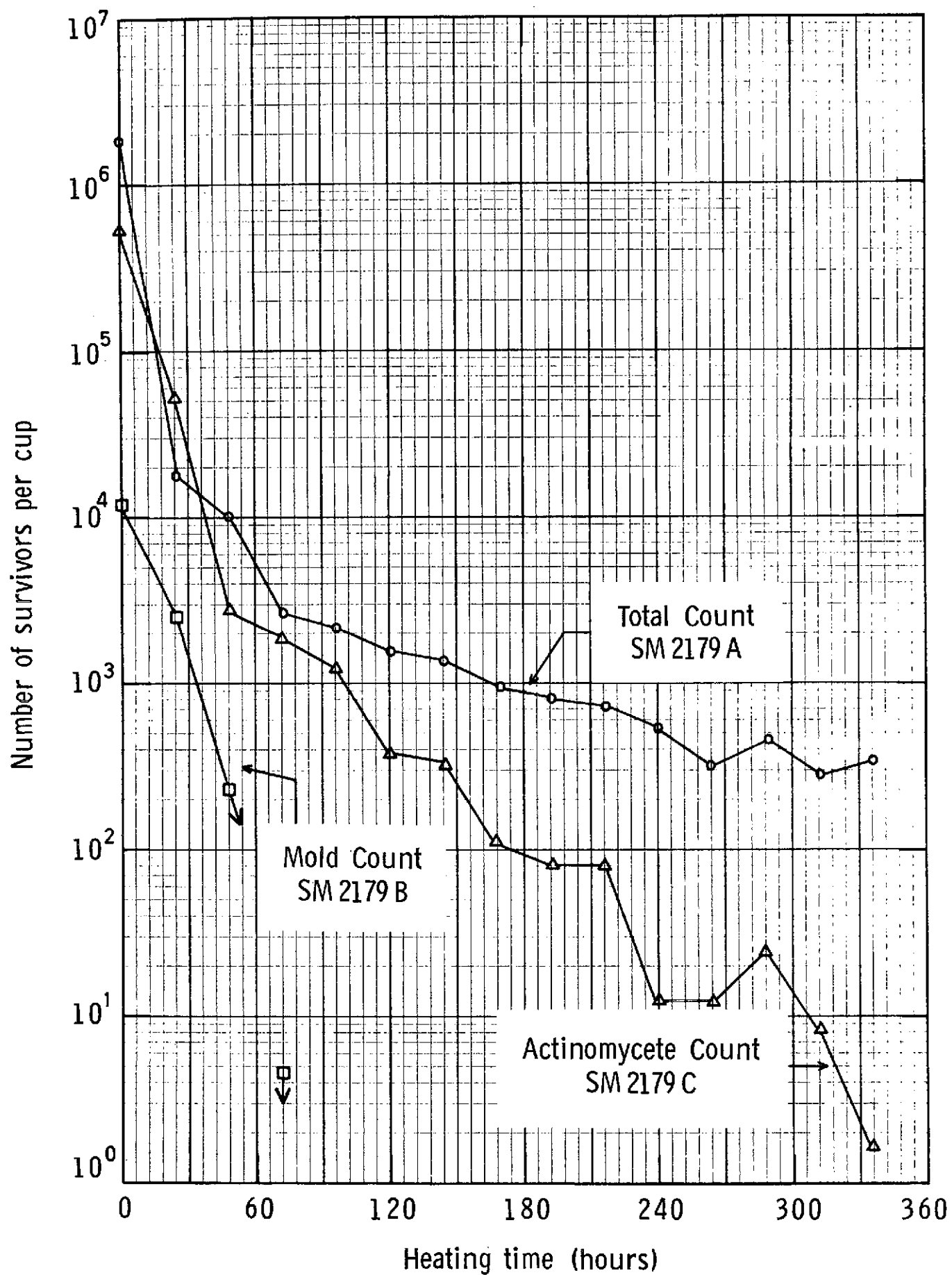
Figure 2.6: Survival characteristics of bacterial, mold, and actinomycete spores associated with dry Dinkytown soil, heated at 110°C.
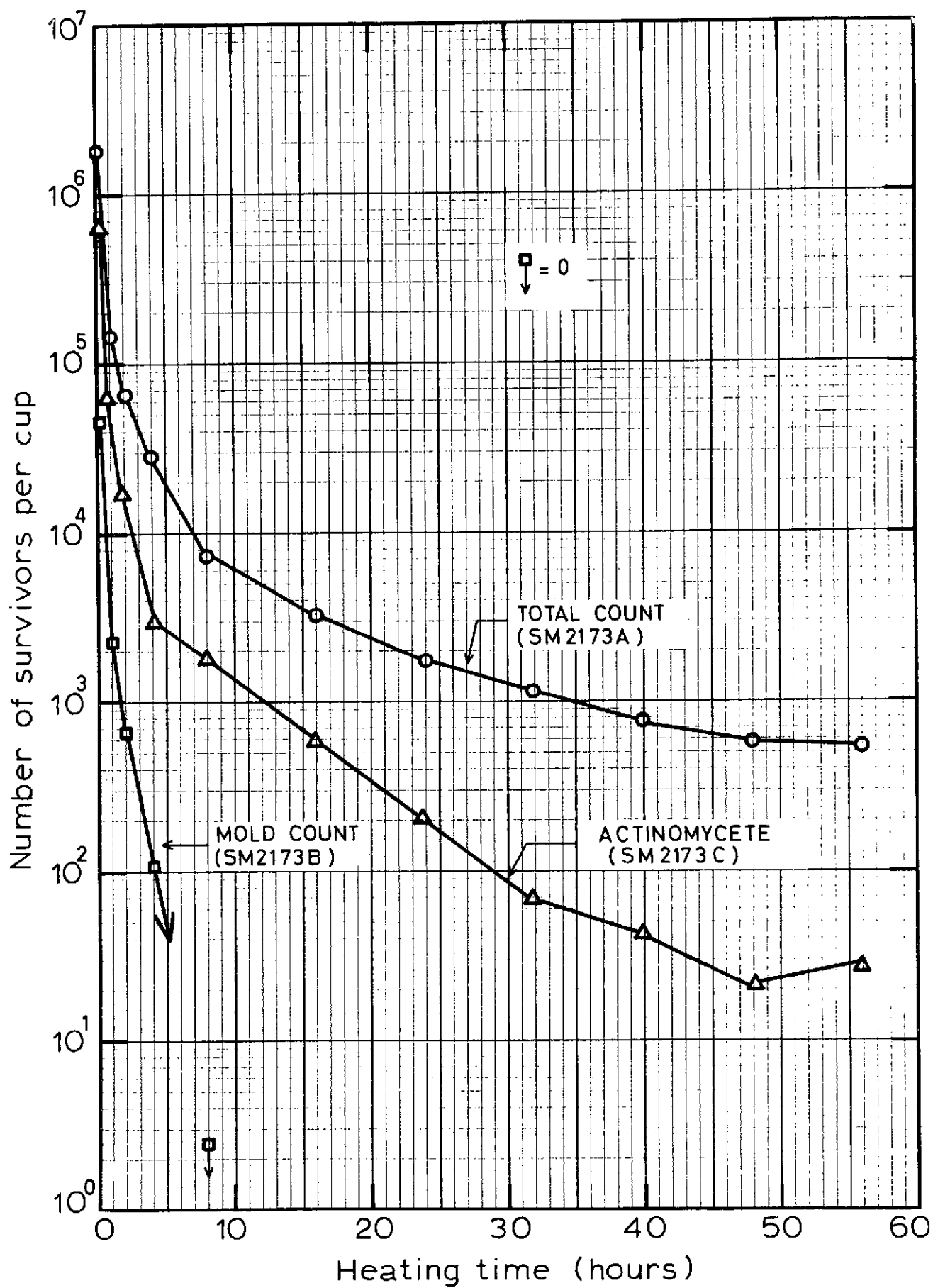
Figure 2.7: Survival characteristics of bacterial, mold, and actinomycete spores associated with dry Dinkytown soil, heated at 125°C.

PROJECT PERSONNEL

The following personnel all made major contributions to this project: Rebecca Gove, Ronald Jacobson, Susan Maki, Irving Pflug, Geraldine Smith, and Yvonne Thun.

DRY HEAT DESTRUCTION RATES OF

BACILLUS SUBTILIS VAR. NIGER IN A CLOSED SYSTEM

Bliss Moore and Irving J. Pflug

Experiments to determine the dry heat destruction rates of Bacillus subtilis var. niger spores at 75 and 90°C at several relative humidities using the heat block system were not completed during this reporting period but extended into the next reporting period during which time the work in this area was concluded. The final report for the heat block studies including the work carried out during this reporting period as well as that during the next reporting period will all appear in the next progress report.

EFFECT OF COMBINED HEAT AND RADIATION ON MICROBIAL DESTRUCTION

Donald A. Fisher and Irving J. Pflug

INTRODUCTION

This is the continuation of a study that was initiated in June 1971. Prior to this reporting period a prototype controlled environmental system for use in this study was designed and constructed. The University of Minnesota Cesium-137 radiation source that will be used in this study was mapped.

During this reporting period the environmental system, which has been designed for use in the gamma facility, was tested and modifications were made to improve its performance. Survivor curves were generated using Bacillus subtilis var. niger spores (AAOE).

OBJECTIVE

The objective of this study is to investigate the synergistic effect which occurs when spores are subjected simultaneously to dry heat and gamma radiation. To accomplish this objective requires that we: 1) investigate the survival of spores on surfaces at various temperatures in a precisely controlled environmental system, 2) determine the rate of destruction of these spores at ambient temperature when subjected to gamma radiation, and 3) determine the rate of destruction of these spores when they are subjected to combined gamma radiation and thermal stress.

TESTS PERFORMED AND RESULTS OBTAINED

Modification of the Environmental System

After the environmental system, described in Progress Report 7, was built, a testing program was initiated to detect its faults. The system was modified and then retested. The following are problems uncovered and the solutions which were found.

Temperature in the environmental chamber. The temperature in the environmental chamber varied as much as 2°C between specimen holders. The source of this uneven heating was traced to the position of the chamber's heaters. These heaters were fastened to the reverse side of the plate on which the specimen holders

were mounted. By mounting the heaters away from this plate, these temperature differences were eliminated.

Relative humidity measurement. At first it was felt that with the flow rates encountered and the insulation used, we could accurately monitor the psychrometric conditions of the environmental chamber. However, there was substantial heat lost from the airstream from the time it left the environmental chamber until it reached the chamber containing the wet and dry bulb thermocouples. This problem was eliminated by measuring the dry bulb temperature in the environmental chamber and using the downstream wet and dry bulb measurements to measure the water vapor pressure or absolute humidity of the airstream. Since the absolute humidities would be the same for each location, we were effectively measuring the absolute humidity in the environmental chamber. This fact was confirmed by temporarily placing a wet bulb in the environmental chamber and comparing the humidity measured at this position with the humidity measured downstream. There was agreement between these two measurements as well as with the saturation temperature in the spray tank.

Flow measurement. There was a relatively high pressure drop across the calibrated orifice through the system that limited airflow. An alternate method of airflow measurement was developed. The electric power input to the heater just downstream of the saturation chamber was monitored along with the air temperature rise across the heater. Since the heat capacity of the airstream is known, the flow rate can be calculated directly using these data.

Biological Testing

Following completion of the above modifications plus a number of minor hardware changes, a destruction rate dry heat test was performed. Bacillus subtilis spores were subjected to an airstream at 90°C and 75% relative humidity without any radiation. Figure 4.1 shows the resulting survivor curves for this experiment.

A parallel test under identical design temperature and relative humidity conditions GS2146A was carried out. The results were of the same general order of magnitude.

The following important results were obtained from these experiments:
1. The survival level at the three separated points seemed to be approximately the same. Dispersion of experimental results is expected at high humidity and the scatter encountered was not unexpected.

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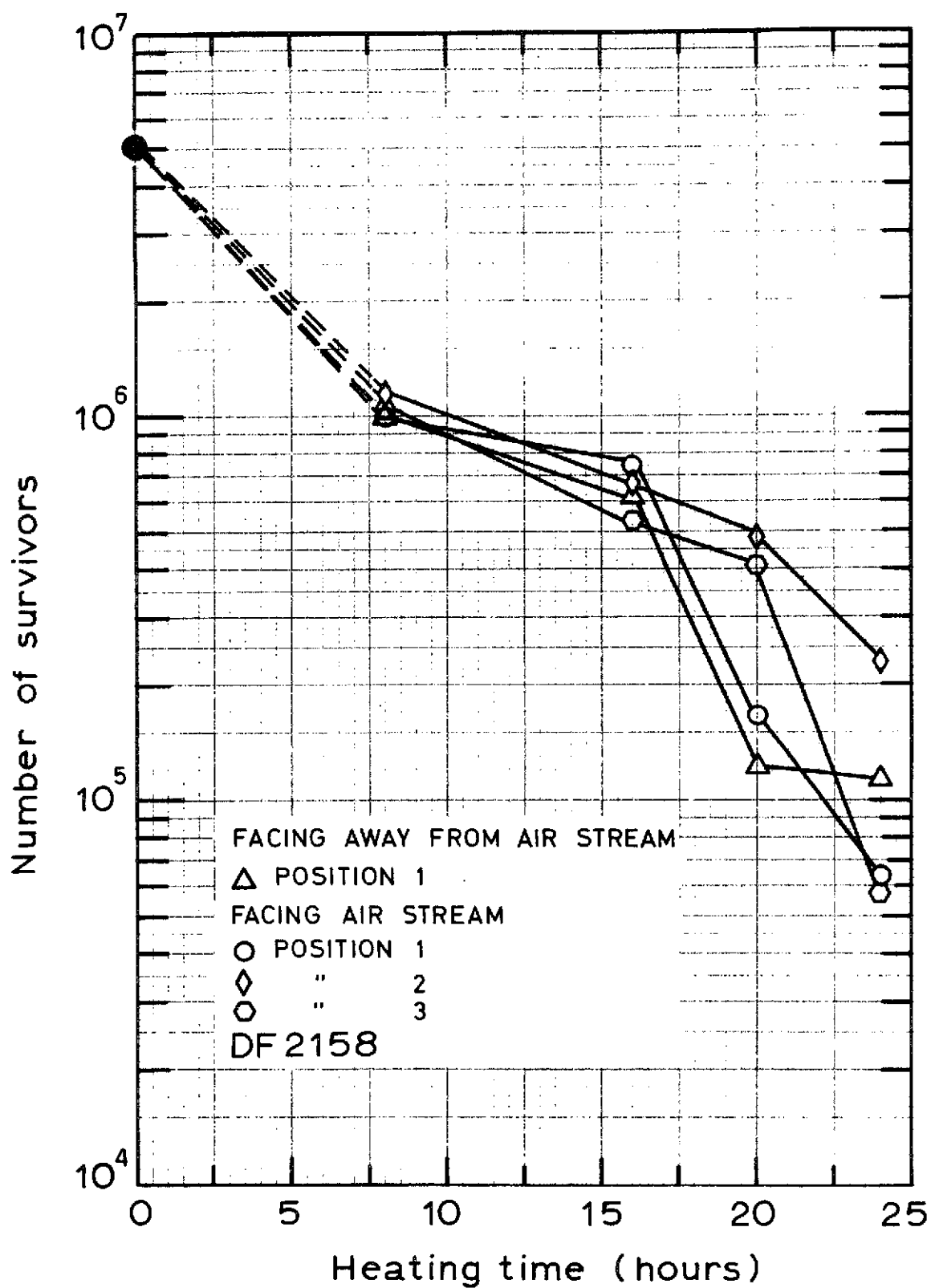
Figure 4.1: Survivor curves for B. subtilis var. niger spores at 90°C and 75 RH at various positions in the environmental chamber of the thermoradiation environmental system.

2. We have shown that it is possible to generate a survivor curve under conditions which are more difficult to control than those that will be encountered in the proposed experimental plan.

3. Two tests were carried out on different days. The results of the two tests were not significantly different.

4. Orientation of the spore strips with respect to the direction of the airstream did not appear to have a significant effect on spore survival.

SUMMARY

The controlled environmental system has been tested, modified, and retested. It has proven to be an operational unit which performs quite closely to the specifications set forth in Progress Report #7. Survivor curve studies have been carried out at 90°C and 75% RH. Additional controlled environmental system components are now being fabricated for carrying out the tests at the several temperature and radiation levels.

FUTURE WORK

As soon as all construction work has been completed, we will begin bacteriological testing with the apparatus. Our test plan is as follows:

1. We plan to generate survivor curves for spores of Bacillus subtilis var. niger subjected only to thermal stress. Temperature levels of 60, 75, 90, and 105°C will be used.

2. We will examine the lethal effect of radiation on spores at ambient temperature using radiation levels of 5, 10, and 20 kR/hour.

3. Survivor curves of spores subjected to combinations of the above-mentioned temperature and gamma radiation levels will be determined.

PROJECT PERSONNEL

The following personnel all made major contributions to this project: Donald Fisher, Irving Pflug, and Stephen Znameroski.

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APPENDIX A: THE RELATIONSHIPS OF DIFFERENT INDICES OF WATER CONTENT IN
DRY HEAT MICROBIAL DESTRUCTION SYSTEMS

Donald A. Fisher, Ronald L. Jacobson, and Irving J. Pflug

Introduction

The dry heat destruction of bacterial spores is a function of the water
content of the spore during heating. Therefore, the D-value or other microbial
destructions rate parameters must be related to a water content parameter--either
water content itself or the relative vapor pressure of the water in the spore.
For a specific spore prepared in a specific manner, there is a rather fixed
relationship between spore water content and the relative humidity of the
atmosphere adjacent to the spore.

Several different indices--relative humidity at test temperature,
relative humidity at ambient temperature, parts per million water, absolute
humidity and dew point--are being used in the NASA Planetary Quarantine
program to quantify water vapor in dry heat microbial destruction systems.
To compare dry heat destruction data, where the concentration of water in
the atmosphere surrounding the spores has an effect on the spore destruction
rate, it is necessary that all the data be in terms of a single water measure-
ment index. Since several indices are being used, it may be necessary to convert
from one water reporting system to another. Since humidity is a material
variable, it cannot be linearly converted from one form to another as can
physical variables such as temperature and pressure. Furthermore, it is
sometimes necessary to specify additional physical parameters before such a
conversion can be made.

The objective in preparing this report was to describe the available
systems and methods of converting data in one measurement system to another
measurement system. In this report the physical basis for each of the water
indices used in dry heat microbial destruction experimentation will be discussed.
The formulae needed to convert from each of the reporting forms to percent
relative humidity are also given.

Description of Water in Spores

Scott (1957) used the term water activity to describe the status of the
water in a solution or substrate. He defined water activity, A_w, as the ratio
of the apparent vapor pressure of the solution or substrate to the saturated

vapor pressure at a given temperature. In his examples, the A_w of solutes in aqueous solutions are calculated using the osmotic coefficient, the molal concentration of the solute and the number of ions generated by each molecule of solute. Theoretically, under equilibrium conditions the water vapor pressure in the gas above and in the solution will be equal.

The water vapor pressure in the gas can be measured by psychrometric methods. The ratio of the water vapor pressure in the headspace to the saturated water vapor pressure is the relative humidity and, at equilibrium, is theoretically equal to the water activity of the solution.

The concept of water activity of solutions adopted by Scott (1957) has been extended to microbial cells and spores. Since it is possible to confuse terms, the following are suggested to clarify the description of water in dry microbial systems: Relative humidity is a real, engineering unit that is the ratio of two measured quantities--the actual water vapor pressure in a system and the saturated water vapor pressure at the same temperature. It is used with gaseous systems, for example, to describe the water condition in the atmosphere surrounding bacterial cells or spores. Water activity, A_w, is a term used to describe the relative water availability inside a microbial cell or spore. It is a theoretical term that cannot be measured. If the cell or spore is in equilibrium with the surrounding atmosphere, theoretically the water activity of the spore is equal to the external relative humidity.

In reporting dry heat research data, if in a microbial destruction rate test the relative humidity is measured and controlled, then the results should be reported as a function of relative humidity, not water activity. Whenever a relative humidity value is reported, the temperature at which the relative humidity was measured should be included; for example, 0.2% RH (110°C).

Properties of Gas Mixtures

Prior to outlining the terms used to describe water content, the physical laws that describe the behavior of gas mixtures will be reviewed.

The physical state of a gas can many times be described by the Ideal Gas Law. The equation of state of an ideal gas is expressed by the following relationship:

$$PV = nRT \tag{1}$$

where P = pressure exerted by the gas

T = absolute temperature

R = ideal gas proportionality constant

n = number of moles of gas

V = volume occupied by the n moles

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In a mixture of gases, the molecules of each component gas are distributed throughout the containing vessel. The total pressure is the sum of the pressures exerted by the molecules of the component gases. By definition, the <u>partial pressure</u> of any one component gas is the pressure that the component gas will exert when it is alone in the same volume at the same temperature. Also by definition, the <u>pure-component volume</u> of a constituent gas is the volume that the component gas alone will occupy at the system pressure and temperature.

For an ideal gas, many properties are additive. The total pressure (P) is equal to the sum of the partial pressures.

$$P = p_a + p_b + p_c + \ldots \tag{2}$$

where p_a, p_b, p_c, etc. are the partial pressures of component gases a, b, c, etc.

This is a statement of Dalton's Law. In the same fashion, the total volume is equal to the sum of the pure-component volumes.

$$V = V_a + V_b + V_c + \ldots \tag{3}$$

where V_a, V_b, V_c, etc. are pure-component volumes of gases a, b, c, etc.

Equation 3 is many times referred to as the Law of AMAGAT or Leduc's Law.

These relationships are required in order to understand the interrelationships between different methods of measuring water in an air atmosphere. For analytical purposes, the mixture of water and air is usually represented as a bi-component mixture of dry air and water vapor where the dry gas is a complex mixture of gases including nitrogen, oxygen, carbon dioxide, and traces of the other gases found in the atmosphere. The molecular weight of the dry gas is taken to be 28.967.

While dry air exhibits the characteristics of an ideal gas, water vapor deviates somewhat from the Ideal Gas Law. However, this deviation is second order in effect.

<u>Description of Water in Gas Mixtures (Humidity)*</u>

There are many distinct humidity forms in use today. We shall describe eight forms that are used to describe the water vapor condition in dry heat atmosphere. All forms can be divided into two groups:

    I.   Expressions based on water substance

       A.   Vapor density

       B.   Relative humidity

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* In preparing this section of the report, "Humidity Reporting Forms and Humidity Sensors" by Elias J. Amdur (1968) has been used extensively.

C. Humidity to gas ratios

    1. Specific humidity (mixing ratio)

    2. Mole fraction

    3. Parts per million

II. Phenomenological forms

  A. Vapor pressure

  B. Dew point

  C. Wet and dry bulb psychrometry

Vapor Density. The gravimetric determination of water vapor in a gas yields data in the form "weight of water per volume of gas" at the temperature of the experiment.

Vapor density is useful because rates of evaporation, diffusion and reaction are related to differences in concentration of the constituents of a system; also because it directly states the quantity of water vapor by weight in a given volume, rather than as a ratio involving other data.

The numerical value of the vapor density for a particular sample varies with both temperature and pressure in open systems such as we ordinarily encounter in most humidity problems. Therefore, it is sometimes cumbersome to handle in computations.

In a closed system the vapor density method is a conservative form maintaining the same value with changes in temperature and pressure. In the dry heat studies carried out at the FDA-Cincinnati Laboratories, humidity data has been reported using this form.

Relative Humidity. The relative humidity form is widely used by the comfort air-conditioning industry and by persons involved in the storage and processing of materials. It is unique among the humidity reporting forms because the moisture content of many materials is a direct function of relative humidity.

Relative humidity is defined in ASHRAE (1972) as the ratio of the mole fraction of water vapor present in the air to the mole fraction of water vapor present in saturated air at the same temperature and barometric pressure. It approximately equals the ratio of the partial pressure or density of the water vapor in the air to the saturation pressure or density, respectively, of water vapor at the same temperature.

The relative humidity form is not conservative with respect to either temperature or pressure in closed or open systems. Since the relative humidity may be defined as the ratio of the moisture content of a space to the saturation

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moisture content, it is affected by temperature (the reference saturation moisture content is temperature dependent). Changes in the pressure of a given sample affect the relative humidity. When there is a change in temperature and/or pressure, the corresponding change in relative humidity can be determined by, (1) converting relative humidity to the defining equation, (2) adjustment of the individual terms, and (3) recombination to determine the new relative humidity.

Humidity to gas ratios. Three methods of expressing humidity as a ratio will be considered in this discussion:

$$\text{Specific Humidity} = \frac{\text{Weight of Water Vapor}}{\text{Weight of Dry Gas}}$$

$$\text{Mole Fraction} = \frac{\text{Moles Water Vapor}}{\text{Total Moles Wet Gas}}$$

$$\text{ppm} = \frac{\text{Volume of Water Vapor}}{\text{Million } (10^6) \text{ Volumes of Wet Gas}}$$

Specific humidity, humidity ratio (ASHRAE 1972), humidity (Zimmerman & Lavine 1964), mixing ratio, and absolute humidity (Perry 1963) are all terms used to describe the ratio of the weight of water vapor to a unit weight of coexisting dry gas. The dry basis ratio is used principally because it is a completely conservative form and lends itself readily to computations where the dry gas fraction does not change.

Mole fraction is the moles of water vapor to the total moles of wet gas. It is an equally conservative form.

Parts per million (ppm) is the ratio of the volume of the water vapor to a million volumetric parts of total gas. Using the Ideal Gas Law, the ppm of water can be related to the partial pressure of water vapor (P_w) in the following manner:

$$\text{ppm} = \frac{10^6 \, P_w}{P} \tag{4}$$

where P is the total pressure

Since it is based on pure-component volume, it also represents moles of water per million total moles of gas and, therefore, a million-fold measure of the mole fraction of water. This measure is also independent of temperature and total pressure barring any phase change. Although this measure is usually employed to indicate the water content of relatively dry gases, it can be used at all water levels up to saturation.

Vapor Pressure. The vapor pressure of water is an absolute humidity form and the rate of evaporation, diffusion, and reaction are proportional to vapor pressure differences as well as to vapor density. The vapor pressure is, in fact, the preferred form for most purposes, largely because it is more conservative. A numerical value for the vapor pressure varies with total pressure in open systems but is not affected by temperature. In closed systems, the value of the vapor pressure depends on both total pressure and temperature.

Dew Point. The water vapor pressure in a system consisting of a vapor space and a plane water surface in isothermal equilibrium is known as the "saturated" vapor pressure of water at that temperature. The saturation vapor pressure of water as a function of temperature is very accurately known. Because of this relationship, it is theoretically possible to determine the vapor pressure in a space by determining the temperature of a plane water surface which is in equilibrium with it.

The dew point determination, however conducted, is an experiment designed to approximate the saturation temperature of the vapor in a space. In the classical procedure one cools a surface until dew forms on it and determines the temperatures at which the dew just forms and just evaporates from the surface. The mean of these two temperatures is regarded as the dew point.

In spite of theoretical difficulties which prevent attainment of high precision, the dew point measurement yields a useful approximation of the saturation temperature and, therefore, the dew point form is a useful alternative method of expressing the absolute humidity. It is also very useful in itself because it indicates the temperature at which moisture will condense on surfaces or clouds will form in a space. This is a uniquely useful property of this reporting form.

Since the classical dew point determination involves bringing a surface to a "dew point" temperature, this type of measurement is independent of the original temperature of the gas being investigated. Since compression or expansion of a gas mixture affects the water vapor pressure, the dew point is also affected by total pressure changes. The dew point and vapor pressure are therefore equally conservative.

Wet and dry bulb psychrometry. The wet and dry bulb psychrometric instrument in the form of the sling psychrometer is probably the most widely used humidity measuring instrument. It is ordinarily considered to be a relative

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humidity instrument, but examination of the simplified basic equation for this instrument indicates that it yields absolute humidity:

$$(e_w - e_a) = C(T_a - T_w) \qquad (5)$$

where e_w = the vapor pressure of the water on the wick

e_a = the unknown ambient vapor pressure

T_a = the dry bulb temperature

T_w = the wet bulb temperature

The two temperatures, T_a and T_w, are read from the instrument. The value of e_w can be determined from a vapor pressure table knowing T_w. Thus, e_a is the only unknown variable. The percent relative humidity may be obtained by dividing e_a by e_w and multiplying by 100. In practice, tables, charts, or accepted empirical equations of RH vs. the wet and dry bulb are generally used.

The constant C is the ratio of the sensible heat transfer coefficient in the particular bulb design used to the vapor transfer coefficient converted to its equivalent value in terms of heat of evaporation. Since this ratio is not a constant unless the air velocity exceeds 900 ft./sec., a motorized blower sucking the air sample over the bulbs yields more satisfactory data than are obtained with a sling instrument.

It should also be noted that C is proportional to the total pressure as found from both theoretical and experimental considerations. Thus for measurements made at pressures significantly different than 1 atmosphere, normal psychrometric charts should not be used. Special nomographic charts for the appropriate pressure should be consulted [Zimmerman and Lavine(1964)].

Wet and dry bulb psychrometry is primarily used as a method of measurement rather than as a reporting form for water vapor in air. Since this method is a measure of the vapor pressure of water, it retains the conservative qualities of vapor pressure measurements.

Water Measurement Conversion to Relative Humidity

Relative humidity is the form of choice for reporting the water vapor characteristics of the gaseous environments surrounding spores during dry heat testing since it is equal to the spore water activity when the spore and surrounding atmosphere are in equilibrium. During non-equilibrium periods, relative humidity again appears to be the proper choice from a diffusional transport viewpoint. Thus, it is desirable to have equations at hand which relate other water measurements to relative humidity.

Our general plan is to discuss water measurement conversion in the same order that we discussed the measurement forms. However, since vapor pressure is basic to other calculations we shall discuss it first, followed by a short discussion of the effect of temperature on relative humidity, before proceeding to the other forms.

Vapor pressure to relative humidity. The ratio of the partial pressure of the water vapor to the saturated water vapor pressure at the same temperature is the relative humidity of the gas. Relative humidity is thus expressed as

$$RH = \frac{p_w}{p_s} \tag{6}$$

where p_w = partial pressure of water vapor
p_s = saturated vapor pressure

The relative humidity of a particular gas sample will be sensitive to temperature and pressure changes. First of all, the partial pressure of the water vapor of a particular gas mixture is dependent on both temperature and pressure. From Dalton's Law and the Ideal Gas Law, we recognize that a change in total pressure of a gas sample effects a proportionate change in partial pressure of the water vapor. From the Ideal Gas Law, we see that a change in temperature changes the partial pressure of water in a closed system (the volume of the gas sample is constant). In an open system a change in temperature does not affect the partial pressure of the water vapor. Lastly, a change in temperature affects the value of the reference saturation vapor pressure used as the denominator of Equation 6.

Ambient relative humidity to treatment RH. When dry heat testing is performed using a hot plate system or an air oven through which ambient air circulates, it is often desirable to know the relative humidity on the basis of the treatment temperature when we know the relative humidity of the atmosphere surrounding the hot plate or circulating through the oven. The equation relating relative humidity at ambient temperature, $RH_{(a)}$, to the relative humidity at test temperature, $RH_{(T)}$, is

$$RH_{(T)} = \frac{RH_{(a)} \, P_{s(a)}}{P_{s(T)}} \qquad (7)$$

where $P_{s(a)}$ = saturation pressure at ambient temperature

This equation holds as long as the total pressure at the ambient condition and the total pressure at the test condition are equal. Garst and Lindell (1970) have developed a chart for the conversion from relative humidity at one condition to relative humidity at another condition. Their chart is reproduced as Figure 1.

When the ambient air is encapsulated in a closed system as the gaseous environment to be heated to test temperature T without any other source of water, the relationship between ambient and treatment relative humidities will be

$$RH_{(T)} = RH_{(a)} \, \frac{P_{s(a)}}{P_{s(T)}} \, \frac{T}{T_a} \qquad (8)$$

where T_a is the temperature of the ambient

Vapor density to relative humidity. From the Ideal Gas Law the partial pressure of water vapor behaves according to the equation

$$P_w = \frac{n_w}{V} \, RT \qquad (9)$$

Thus, percent relative humidity can be linearly related to a given concentration measure:

$$\%RH_{(T)} = \frac{P_w}{P_{s(T)}} \times 100\% = \frac{n}{V} \, \frac{RT}{P_{s(T)}} \, 100\% \qquad (10)$$

or more generally

$$\%RH_{(T)} = C \, \frac{T}{P_{s(T)}} \, \delta \qquad (11)$$

where C = concentration of water (units specified in Table 1)

    δ = constant of proportionality which depends on units of C (see Table 1)

    $P_{s(T)}$ = saturation pressure of water at temperature T in mm Hg

    T = temperature (°K)

Table I

Definition of Values for Equation 11

units of C	δ
$\dfrac{moles}{liter}$	$62.37 \ \dfrac{mm \ Hg}{^\circ C} \ \dfrac{liter}{gm-mole}$
$\dfrac{gm}{ml}$	$3.46 \times 10^3 \ \dfrac{mm \ Hg}{^\circ K} \ \dfrac{cm^3}{gm}$
$\dfrac{lb_m}{ft^3}$	$9.59 \times 10^4 \ \dfrac{mm \ Hg}{^\circ K} \ \dfrac{ft^3}{lb_m}$

Specific humidity to relative humidity. The absolute humidity, w, can be calculated from

$$w = \frac{w_{water}}{w_{air}} = \frac{18.016 \ p_w}{28.967(P-p_w)} \tag{12}$$

where w_{water} = weight of water vapor per unit volume

w_{air} = weight of dry air per unit volume

p_w = partial pressure of water vapor

P = total pressure

18.016 = molecular weight of water vapor

28.967 = molecular weight of dry air

If the water content is given in terms of absolute humidity, w, we can calculate $RH_{(T)}$ using the following equation:

$$RH_{(T)} = \frac{28.967 \ w \ P}{P_{s(T)} \ (18.016 + 28.967w)} \tag{13}$$

where w = humidity as lb. of water vapor per lb. of dry air

Parts per million to relative humidity. An equation relating water content expressed as ppm of water to relative humidity at a temperature can be obtained

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by combining equations 4 and 6. The resulting equation is

$$\%RH_{(T)} = \frac{ppm \times P}{10^4 \times P_{s(T)}} \tag{14}$$

For an open system where the total pressure is constant at one atmosphere, this relationship is linear and equation 14 becomes

$$\%RH_{(T)} = \beta_{(T)} \, ppm \tag{15}$$

where the temperature dependency of $\beta_{(T)} = \dfrac{760}{10^4 \, P_{s(T)}}$ can be found in Table 2.*

Table 2

Values of $\beta(T)$ at Various Test Temperatures

T°C	$P_{s(T)}$ [mm Hg]	$\beta(T)$
22	19.80	3.827×10^{-3}
45	71.83	1.058×10^{-3}
60	149.35	5.088×10^{-4}
75	289.05	2.629×10^{-4}
90	525.78	1.445×10^{-4}
100	760.00	1.000×10^{-4}
110	1074.56	7.072×10^{-5}
115	1267.98	5.993×10^{-5}
125	1740.93	4.365×10^{-5}
135	2347.26	3.237×10^{-5}

For a closed system that is sealed at ambient pressure and temperature (T_a), the relationship at other temperatures (T) is more complicated since pressure will also change with temperature. The final total pressure will be the sum of the partial pressures of the component gases. For the dry gas

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* The total pressure was taken as 760 mm Hg or 1 standard atmosphere. This total pressure varies depending on weather and location.

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component, the final partial pressure, $P_{dg(T)}$, upon being heated, increases
in accordance with

$$P_{dg(T)} = P_{dg(a)} \frac{T}{T_{(a)}} \tag{16}$$

where $P_{dg(a)}$ = the partial pressure of the dry gas at the time of
sealing

This equation is only valid for closed systems where the gaseous volumes remain
constant. By definition of relative humidity, the partial pressure of water
at test temperature is

$$P_w = \frac{RH\% \ (T)}{100} \ P_{s(T)} \tag{17}$$

Presumably, the relative humidity at test temperature ($RH\%_{(T)}$) is calculatable
from the amount of liquid water added (see equation 11), and/or the relative
humidity of the ambient gas (see equation 8) which is sealed in the closed
system. At any rate, the total pressure will be

$$P = P_{dg(a)} \frac{T}{T_{(a)}} + \frac{RH\% \ (T)}{100} \ P_{s(T)} \tag{18}$$

Example

Thus, for a closed system having been sealed at 22°C with the dry air
having a partial pressure of 760 mm Hg, the total pressure at test temperature is

$$P = 760 \frac{T}{295} + \frac{RH(\%)}{100} \ P_{s(T)} \tag{19}$$

By combining equations 14, 17, and 19 the relationship necessary to convert
from ppm to %RH is

$$\%RH = \frac{\frac{T}{295} \ \beta(T)ppm}{1 - \frac{ppm}{10^6}} \tag{20}$$

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Figures 2 and 3 respectively, show the relationship of water in ppm to %RH for an open and for a closed system. The results are linear for the open system and nonlinear for a closed system, especially at the larger values of ppm.

Dew point to relative humidity. Some researchers report water content in terms of dew point or the temperature at which condensation starts upon an isobaric cooling (cooling at constant total pressure) of the gas mixture. Since the pressure at dew point equals the partial pressure of water vapor, relative humidity can be calculated as

$$RH_{(T)} = \frac{P_{s\ (DPT)}}{P_{s(T)}} \tag{21}$$

where $P_{s(DPT)}$ = saturation vapor pressure at the dew point temperature.

Figure 1 shows the dew point temperature for gas mixtures of varying temperatures and relative humidities.

Psychrometric forms to relative humidity. Wet and dry bulb psychrometry forms are basic to most common humidity measurements. Conversion of wet and dry bulb temperatures to relative humidity relies heavily on use of psychrometric charts (ASHRAE 1972, Zimmerman and Lavine 1964, et al). Such charts generally describe, for the user's information, the proper technique to determine relative humidity for any psychrometric determination.

SUMMARY

The common methods of measuring water in water-air systems are described. Equations are developed that make possible the conversion of data from one type of measuring unit into percent relative humidity. The location of the relevant conversions can be found by consulting Table 4, and an index of terms defined in the text is contained in Table 5.
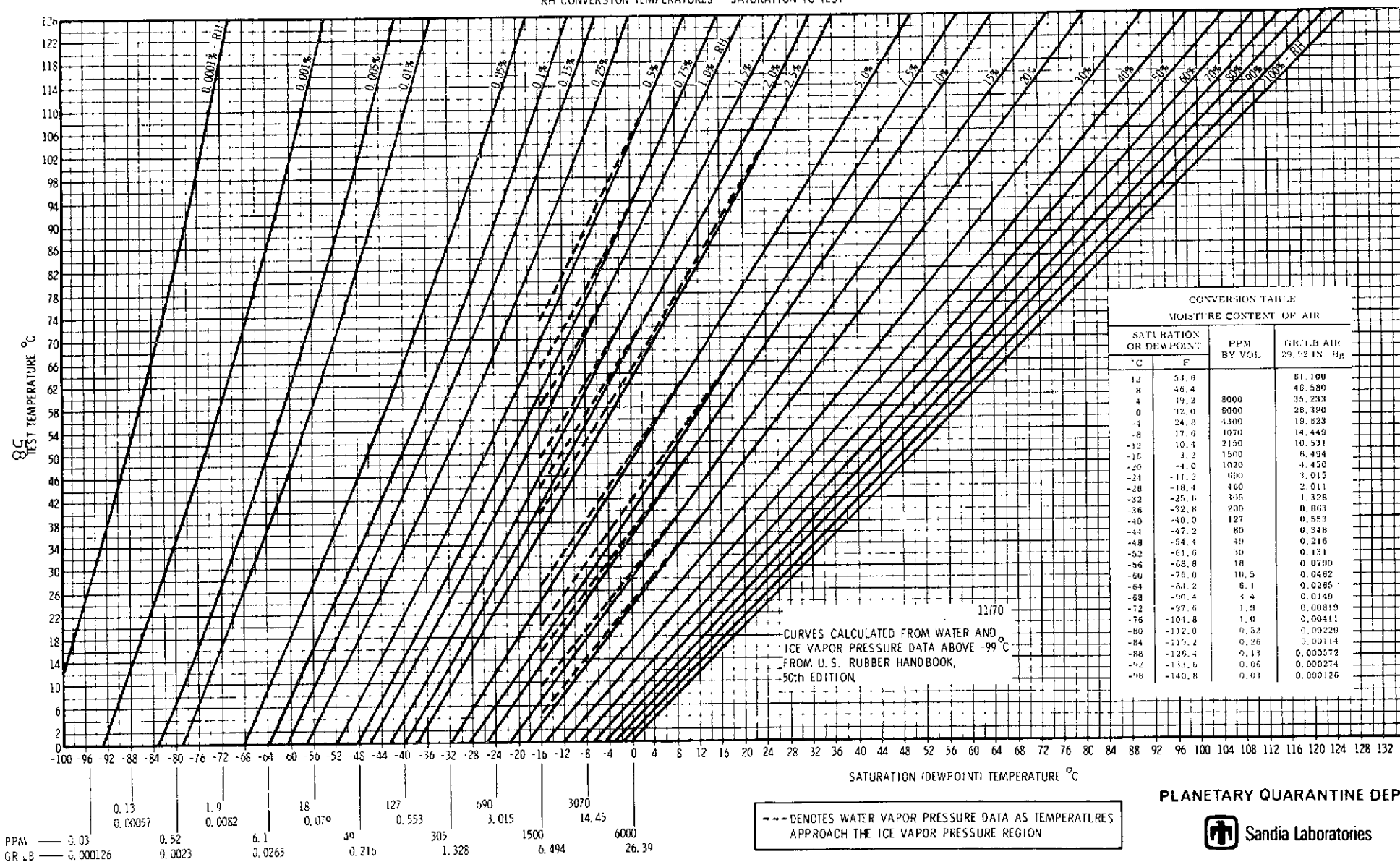
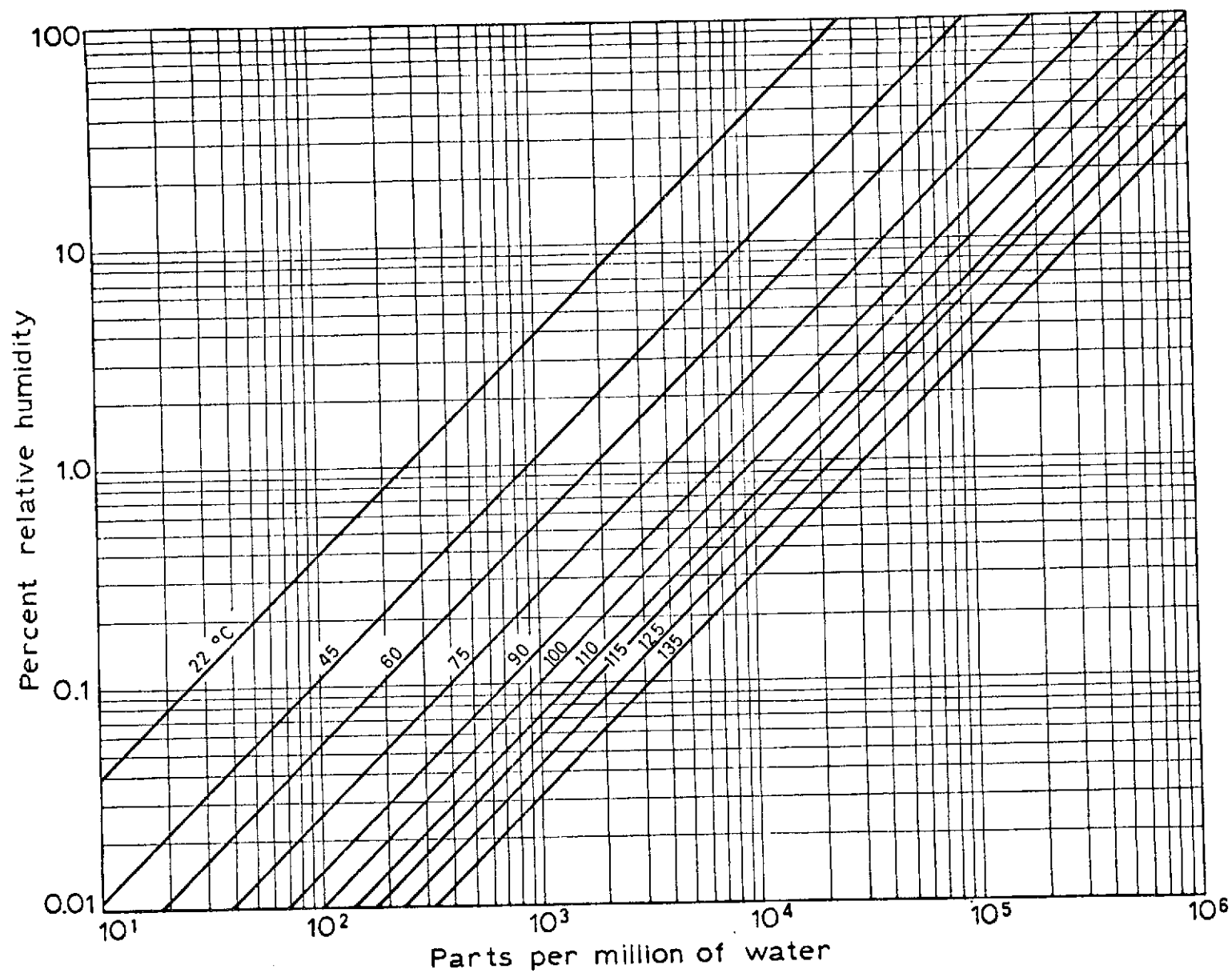Figure I - Relative humidity conversion graph (Taken from Garst & Lindell, 1970)

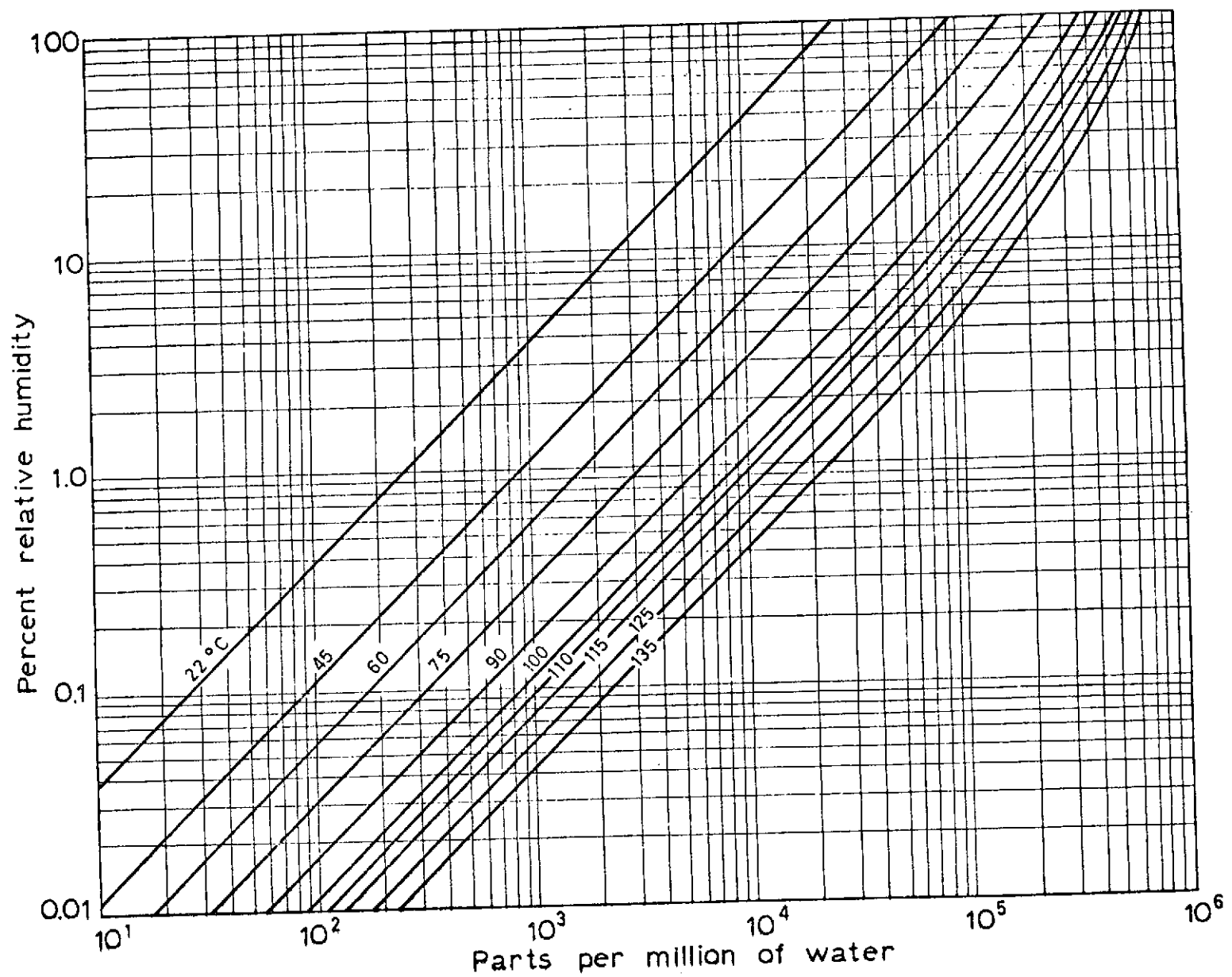Figure 2 – Conversion graph for parts per million of water and relative humidity for an open system (P = 760 mm Hg)

Figure 3 - Conversion graph for parts per million of water and relative humidity for a closed system (p_{dg} = 760 mm Hg)

Table 4

Summary Chart

| To convert from this measure ↓ | → To %RH at treatment temperature | |
	(in open system)	(in closed system)
	[Equation #]	[Equation #]
Ambient RH	7	8
Dew Point	21	21
Parts Per Million	15	20
Specific Humidity	13	13
Vapor Density	11	11

Table 5

List of Terms Defined in Text*

Term	Location
Absolute humidity (Specific Humidity)	5
Ambient relative humidity	8
Dew point	6
Humidity	5
Humidity ratio (Specific Humidity)	5
Ideal Gas Law	2
Mixing ratio	5
Mole fraction	5
Partial pressure	3
Parts per million	5
Pure-component volume	3
Relative humidity	4
Saturation temperature	6
Specific humidity	5
Treatment relative humidity	8
Vapor pressure	6
Water activity	1

*Table 5 lists terms used in this discussion, as well as the point in the text where the definition of each term can be found.

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APPENDIX B: BIOLOGICAL INDICATORS FOR MONITORING STERILIZATION PROCESSES*

Irving J. Pflug, Jacob E. Bearman, and Ronald L. Jacobson

The monitoring of sterilization processes is a challenging problem. To be considered a sterilization process, the probability that the item subject to the sterilization cycle is sterile should be of the order of at least .99900 and in most cases, .999900 to .999999. Since we cannot directly assay for this probability level of sterility and because it is of vital importance that we adequately sterilize objects for use in the health industries, we must have reliable methods of monitoring sterilization processes. Biological indicators (BI), if properly used, are very effective quality control tools for sterilization processes.

A sterilization effect can be monitored through the interpretation of its effect on a physical, chemical, or biological system. Biological indicators fall into three general groups depending upon whether the source of the sensitive agent is, (1) a natural microbial contamination of the product, (2) a material such as garden soil with its natural microbial contamination, or (3) a specific quantity of laboratory-produced microorganisms (usually bacterial spores) which have been inoculated into or onto the product or a carrier which is usually a filter or chromatographic paper strip. In this discussion, we will be considering biological indicators in general with emphasis on one specific type of biological indicator. However, any type of biological indicator can be used that meets the process monitoring requirements.

The use of biological indicators is not new. The "inoculated experimental pack" procedure has been used in the food industry (Townsend et al., 1956 and Hersom and Hulland, 1963) for several decades. Spore papers for use in testing sterilizers have been reported by Kelsey (1958 and 1961). Recently Laskaris and Chaney (1969) evaluated commercially available biological indicators for monitoring autoclave sterilization. The purpose of this paper is to outline some of the quantitative aspects of biological indicators, their design, production, and use.

How do we monitor a wet heat sterilization cycle?

In this presentation we are directing our discussion toward the problem
of monitoring sterilization processes from a manufacturer's quality assurance
standpoint. We realize that the regulatory agencies have sterility monitoring
needs and also that the user of sterile items and devices has a need to be
able to ascertain the sterility level of these items. However, these two
applications will not be treated directly in this presentation.

To monitor a sterilization cycle we must measure the sterilization
effect delivered throughout the load of objects to be sterilized. Since the
rate of heat transfer or steam penetration may not be uniform throughout the
load, we will want to be sure to monitor those areas that heat slowest or are
most remote in terms of steam penetration. Since the effectiveness of a heat
sterilization process is a function of the integrated effect of time and
temperature, the monitoring system must be capable of either carrying out this
integration or else providing the data for the integration to be done as a
separate operation.

We can physically monitor a sterilization process by measuring the time
and temperature variables during a process. We can measure temperature simply
as a function of process time either continuously or at regular time intervals.
The temperature can be monitored during the heat sterilization cycle by placing
a temperature sensing device, usually a thermocouple, in that zone in the load
that will heat most slowly. The procedure of inserting thermocouples is time
consuming and requires a specialist for the correct use of the temperature
measuring probes and the integration of the time-temperature data. In addition,
it is possible that the insertion of the temperature sensing device in the
correct location might destroy the future integrity of the sterilized package.

An alternative to the actual measurement of the physical parameters of
time and temperature is the use of a device which is sensitive to both
temperature and time. This can be inserted into the load prior to placing
the load in the autoclave and removed from the load at the termination of the
cycle. This device is then read to determine if the cycle has met specifica-
tions. Indicators can be either chemical or biological. Browne's Tubes are
chemical indicators; these are sealed glass tubes which contain a red liquid
which, upon heating, changes to amber. When the sterilization is complete it
changes to green (Meynell and Meynell, 1965). Biological indicators generally
utilize calibrated bacterial spores deposited on filter paper strips.

It is important that we recognize that we are discussing an indicator

system. This means not only the bare device but a specific calibration so that the end result is a true acceptance or rejection of the load based on the design criteria except for known probabilities which are at our disposal to specify.

Why use a biological indicator?

Biological indicators have more commendable performance and economic attributes than either physical or chemical monitoring systems. The biological indicator responds to the effect of time at a temperature and therefore gives a plus or minus answer directly to the design criteria. It is, after, all, a direct measure of the effectiveness of the sterilization process including all unknown as well as known factors influencing sterility.

The temperature coefficient of the biological indicator is of the same order of magnitude as the temperature coefficient of the microbial contamination of the load being sterilized. Therefore, the biological indicator can accurately sense the effect of temperature both above and below the design temperature level.

Biological indicators require no mechanical connection to the sterilizer; therefore, they can be inserted into the load prior to moving the load to the sterilizer and removed from the load after it has been removed from the sterilizer. Biological indicators are small; therefore, they can be placed directly into packages or in devices to be sterilized. Biological indicators are also relatively inexpensive.

How and where does the biological indicator fit into the sterilization system?

In Fig. I we have shown a diagram of our interpretation of how we should proceed in the design and control of heat sterilization processes. The types and numbers of microorganisms in the load to be sterilized and the rate of heating of the slowest heating zone in the load are the primary factors to be considered in the design of a sterilization process. Using these data, the sterilization process is designed; the design for a heat sterilization process will be in terms of time at a temperature for the specific situation.

The next phase in the overall sterilization sequence is the carrying out of the sterilization operation. To be sure that the sterilization process design is being met, the actual process must be monitored. An automatic auto-clave will have temperature and time controls to operate the unit. It should also have a second system to monitor the time and temperature of the apparatus during the sterilization cycle. There should be a second system to monitor the load of product being sterilized. The sensor of the product monitoring

system is placed in the load to be sterilized to measure the actual time at a temperature in the slowest heating zone in the load.

The records of the physical measurements of time, temperature, and pressure as well as the data from the biological and chemical indicators will constitute the data for verifying the performance of the sterilizing device.

The role of the biological indicator is to monitor the performance of the sterilizing system as far as the product load is concerned; that is, to ascertain whether or not the designed time at a temperature sterilization cycle has been delivered to the product being sterilized at the point of location of the biological indicator.
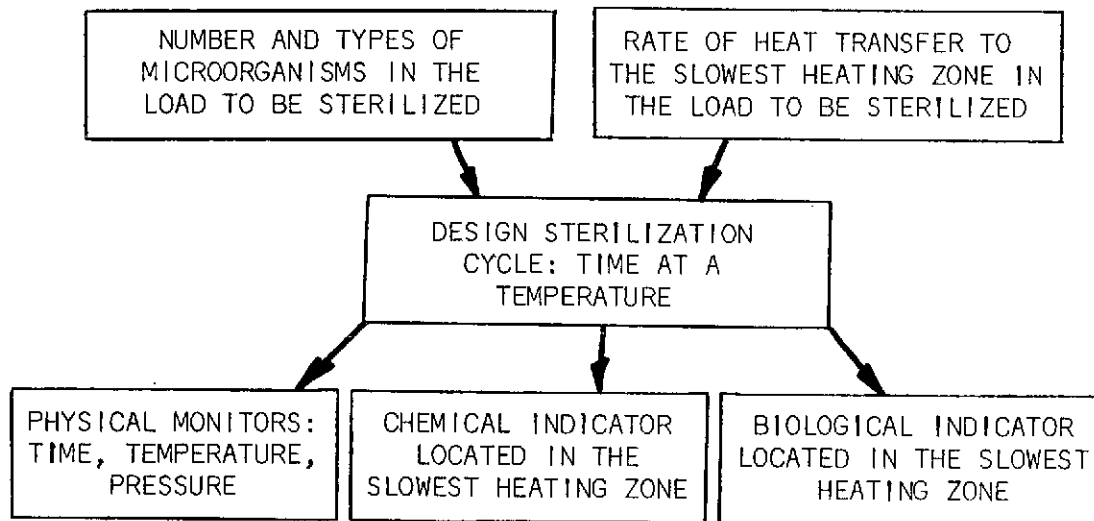
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┌─────────────────────────┐   ┌─────────────────────────┐
│ NUMBER AND TYPES OF     │   │ RATE OF HEAT TRANSFER TO│
│ MICROORGANISMS IN THE   │   │ THE SLOWEST HEATING ZONE IN│
│ LOAD TO BE STERILIZED   │   │ THE LOAD TO BE STERILIZED│
└─────────────────────────┘   └─────────────────────────┘
              ↘                   ↙
         ┌──────────────────────────┐
         │ DESIGN STERILIZATION     │
         │ CYCLE: TIME AT A         │
         │ TEMPERATURE              │
         └──────────────────────────┘
          ↙            ↓            ↘
┌──────────────────┐ ┌──────────────────┐ ┌──────────────────┐
│ PHYSICAL MONITORS:│ │ CHEMICAL INDICATOR│ │ BIOLOGICAL INDICATOR│
│ TIME, TEMPERATURE,│ │ LOCATED IN THE   │ │ LOCATED IN THE SLOWEST│
│ PRESSURE         │ │ SLOWEST HEATING ZONE│ │ HEATING ZONE     │
└──────────────────┘ └──────────────────┘ └──────────────────┘
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Figure I: Diagram showing the different steps in a sterilization process

It is the responsibility of the sterilization group to continuously monitor not only the performance of the sterilization process (using physical, chemical and biological monitors) but also the microbial loads of objects being sterilized. This is the only way to assure that the integrated resistance of the microorganisms in the load to be sterilized does not change.

Changes in the numbers of microorganisms (going from A to B in Fig. 2) and changes in the resistance of the critical group of microorganisms in the load, either due to the mutation of an old species or the arrival of a new more resistant species resulting from a change in the manufacturing process (going from A to C in Fig. 2), will require an adjustment in the design sterilization cycle.

A change in the design sterilization cycle will require a new biological indicator. However, we again want to stress that in our opinion the system must be considered as two separate problems: (1) the design of the sterilization cycle based on the nature of the product being sterilized and its microbial load, and (2) monitoring the delivery of the sterilizing effect using physical, chemical, and biological indicators. These two problems interface but they must be kept separate.

In our opinion, it is wrong to try to directly relate microorganisms in the load to be sterilized to those microorganisms on the biological indicator. The biological indicator can only be calibrated in terms of physical parameters and this calibration is real, predictable, and reliable. Relating the microorganisms in the load to the microorganisms on the spore strip will, we think, obscure the fundamental relationship of the biological indicator to the design sterilization cycle.

Microorganisms used in biological indicators

The choice of species of spores for use in a biological indicator system will depend on the sterilization process to be monitored. For each sterilization process we have the alternative of using a small number of very resistant spores or a large number of less resistant spores. I will show below that as the D-value of the spores of a biological indicator decreases, the biological indicator becomes more sensitive. Listed in Table I are the names, D and z-values of several species of spores that have been used as indicator organisms for wet and dry heat.

A good indicator organism should yield spores that are stable both in heat resistance and in number. The methodology of spore production should be simple and should result in a high percentage of uniform spores. Spore crops of the same organism produced in a similar manner should have the same heat resistance. After exposure to a sterilization cycle there should be rapid germination and growth of the spores using simple media and normal (aerobic) incubation conditions.
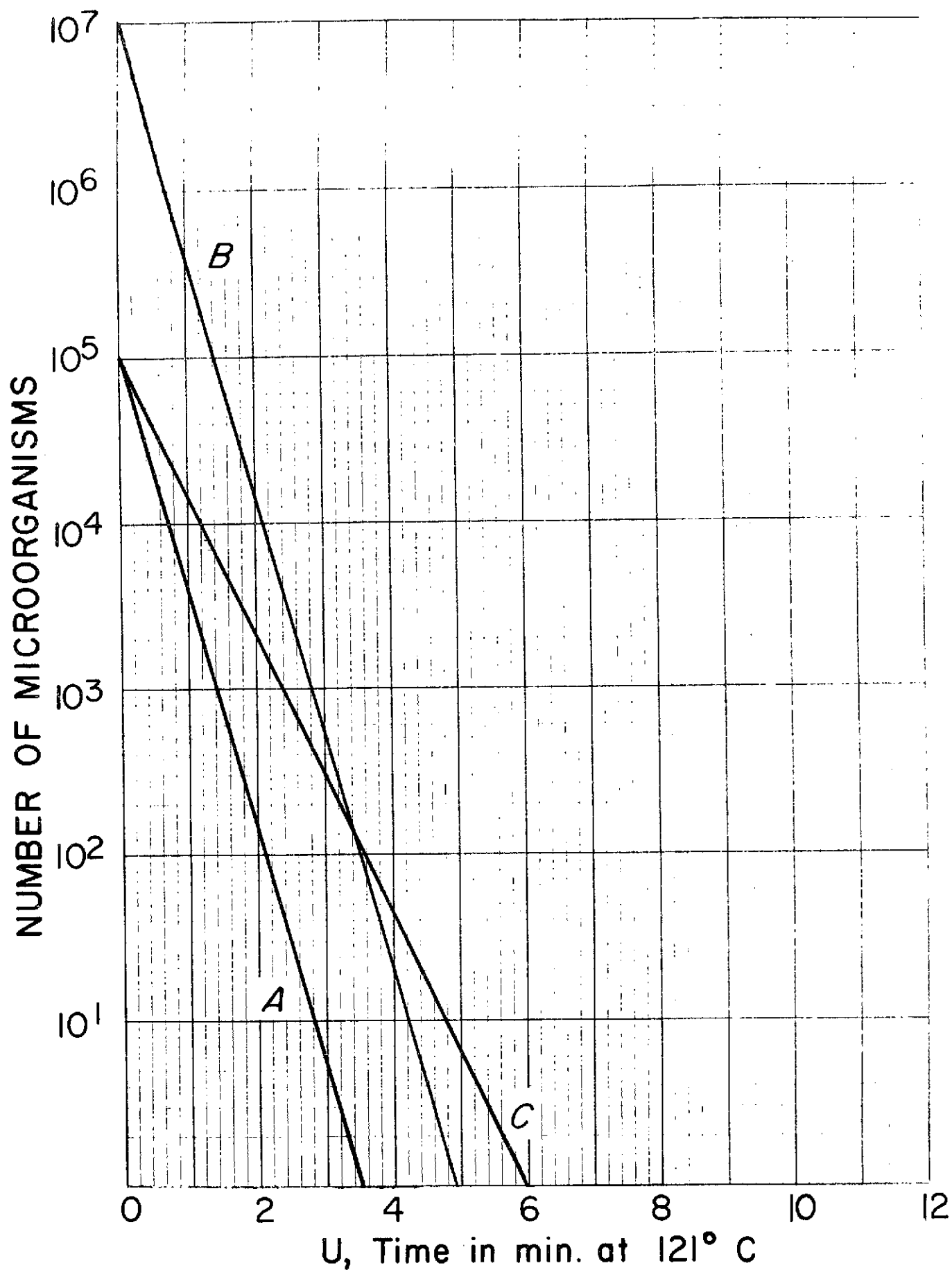
Figure 2: A graph to show the effect of a change in the initial numbers of microorganisms A to B and in the resistance level of microorganisms, A to C, on the time to sterilize.

Table I

Species of Spores that Have Been Used as Biological Indicators

Wet Heat (saturated steam)		
	D_{120} minutes	$z°C$
Bacillus subtilis 5230	0.2 - 0.5	9.5
Clostridium sporagenes (PA3679)	0.8 - 2.0	10
Bacillus stearothermophilus	3.0 - 6.0	10
Dry Heat		
	D_{120} minutes	$z°C$
Bacillus subtilis 5230	50 - 295	18.3
Bacillus subtilis var. niger	36 - 240	21.0

The biological indicator system

In recent years quantitative microbiology has advanced significantly both in theory and in practice. It appears desirable that we use the information developed in quantitative microbiology where it is applicable to biological indicators.

We believe that the assumption should be made that when a large quantity of homogeneous bacterial spores are subjected to a constant heat stress, the number of survivors will decrease logarithmically according to the equation

$$\log N_u = \frac{-U}{D_T} + \log N_o$$

where

N_u = the number of spores surviving after a sterilization treatment of time U at T°C

U = heating time at T°C

D_T = the decimal reduction time at T°C, i.e., the time to reduce the microbial population by 90%

N_o = the initial number of spores subjected to the heat process

Assuming a mechanism of death of bacterial spores which is logarithmic in nature implies that any "tailing effect" that is sometimes observed in survivor curves has been considered negligible. The assumed model is said to define

the means of various populations of surviving spores at <u>different heating times</u> when the estimated values of the unknown parameters of the model are in fact true. However, the estimation procedure and the distribution characteristics of the various populations imply that the <u>results obtained</u> when spores are used as biological indicators are probabilistic and not absolute. We <u>cannot</u> design the spore strip system so that a fifteen minute sterilization process at 121°C will always give zero positive spore strips; whereas, a sterilization process significantly shorter than fifteen minutes, for example twelve minutes, will result in positive spore strips 100% of the time.

In our opinion, a biological indicator system will always involve the use of more than one spore strip. The accept-reject decision will be based on the total number of spore strips used in the load to be sterilized. In this presentation we will assume that ten spore strips are used per sterilizer load (n = 10). It is important that we understand and distinguish between the initial number of spores in the biological indicator system (N_o) and the initial number of spores per spore strip (N_{ss} is the average number of spores per spore strip where $N_{ss} = N_o/n$).

Analytically we determine N_o for the biological indicator system using the sterilization design conditions of the integrated time by temperature or its square wave equivalent. Next we decide on the tolerable probability that the indicator yields a positive result when in truth the desired time and temperature conditions have been obtained. Given this information, we determine the required N_u. We calculate N_o knowing (for the "square-wave" temperature, T) the D_T-value of the test organism and the required N_u. In our example, the sterilization design conditions are fifteen minutes at 121°C. The probability of a positive result is set at 0.05 which implies that $N_{15\ min.}$ = 0.05 organisms. For the test spores, the D_{121}-value is 2.0 minutes, and we determine that N_o is 1.62×10^6. Since we are assuming n = 10, and $N_{ss} = \dfrac{N_o}{n}$, N_{ss} will be 1.62×10^5. Fig. 3 shows the survivor curve for the conditions of this example.

The probability of obtaining a positive spore strip result, i.e., one or more strips positive, as a function of the number of minutes at 121°C is shown in Fig. 4. If the sterilization cycle is only thirteen minutes, the probability of rejection is .40. When the sterilization cycle is seventeen minutes, the probability of rejection is .005. In this example we observe that the biological indicator does not give us a sharp accept-reject answer.
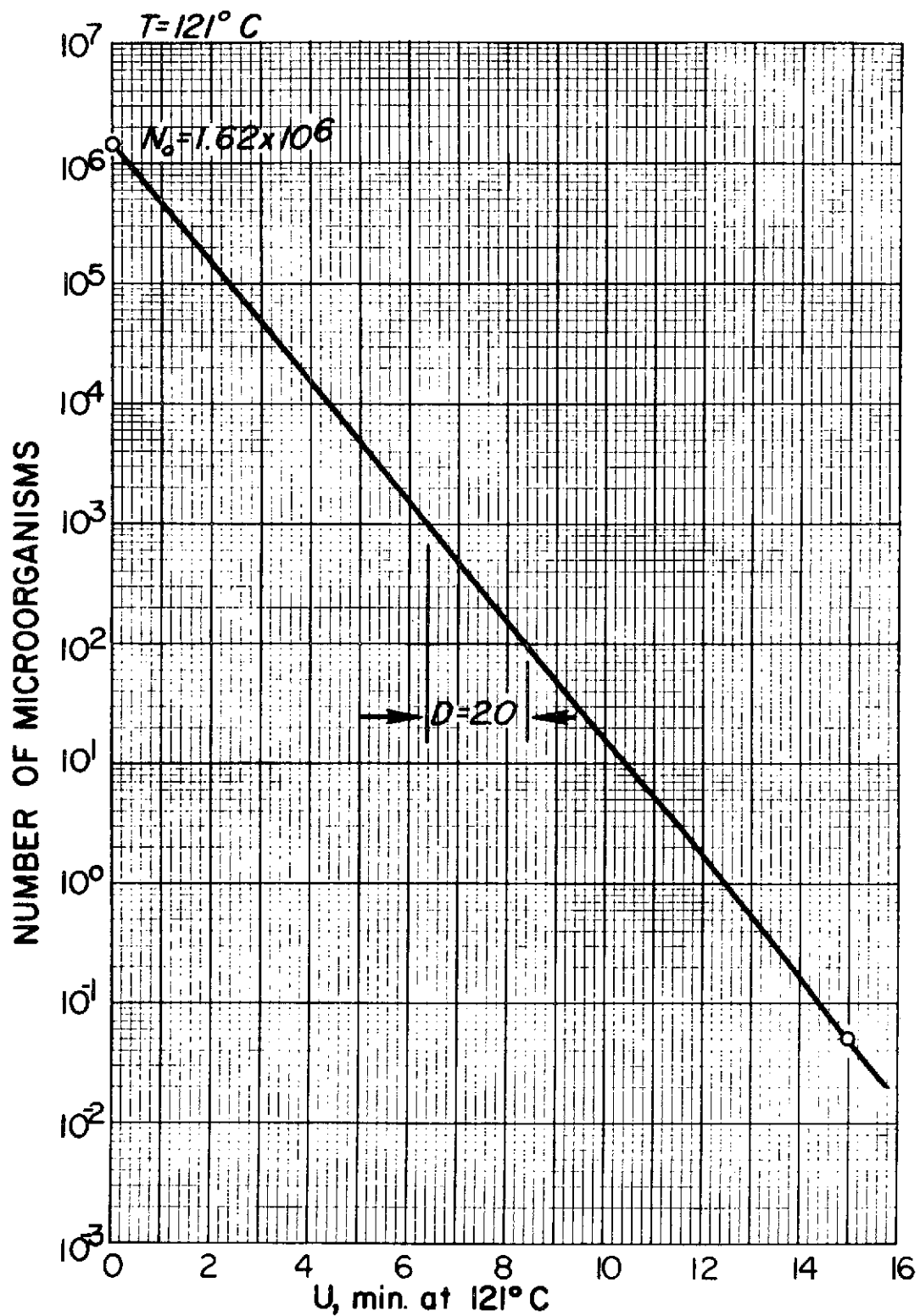
Figure 3: A biological indicator design curve for the conditions: U = 15 minutes, D = 2.0 minutes, N_u = 0.0513 (.05 probability of being positive) which requires an N_o of 1.62 x 10^6 spores.
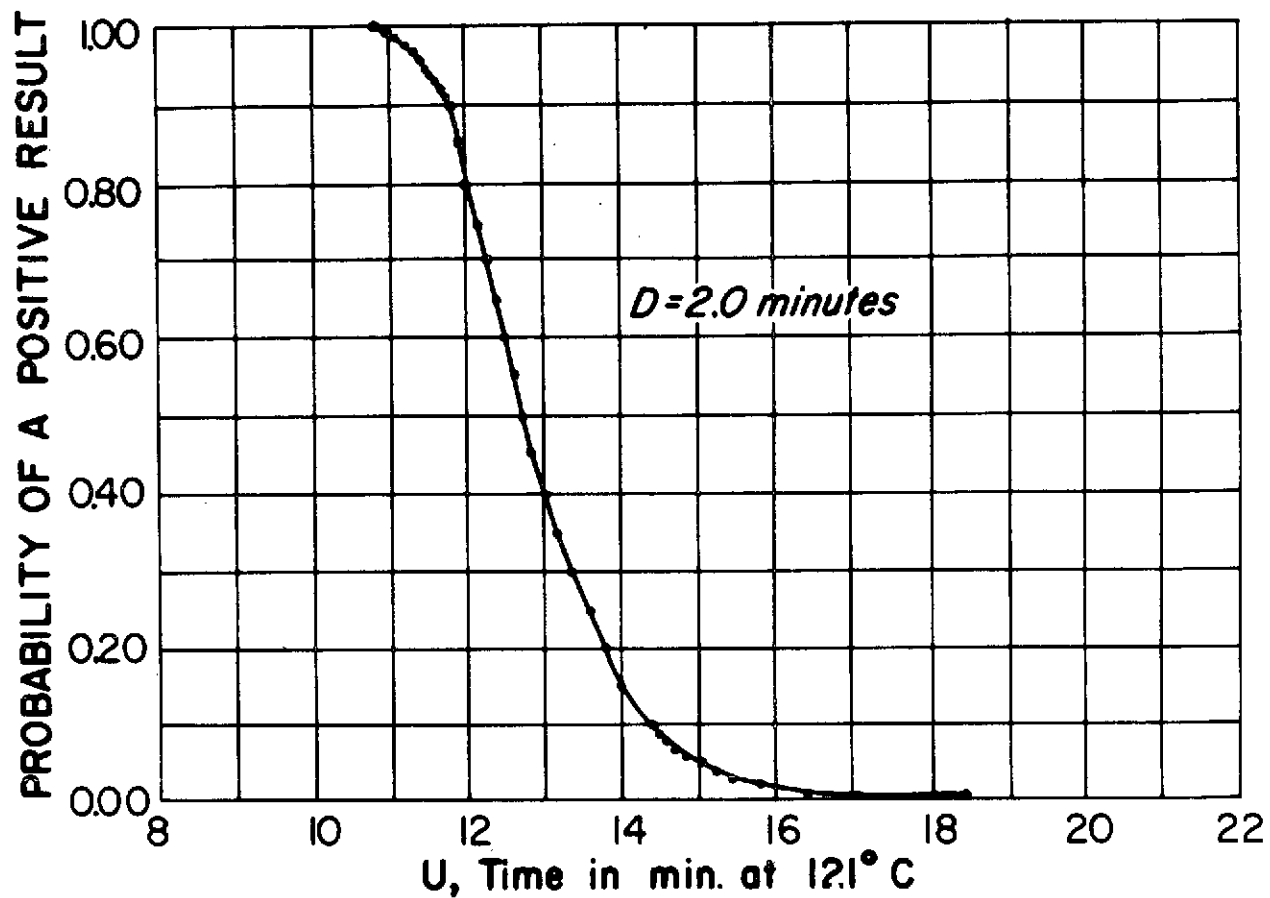
Figure 4: A graph of the probability of a positive result as a function
of heating time U for spores having $D_{121°C}$-values of 2.0 minutes based
on a sterilization cycle design of 15 minutes at 121°C.

However, if properly used and interpreted it is not only adequate, but we believe biological indicators are one of the best methods available for monitoring sterilization processes. In fact, we have a much more realistic system than otherwise.

Fig. 5 illustrates the change in the probabilistic response with changes in the D-value of the spores. We observe that using spores with a D_{125}-value of 2.0 minutes, we obtain a much sharper change in response as a parameter of time than with spores that have a $D_{125°C}$-value of 5.0. From these results, it is apparent that spores with the smallest D-value should be used within the constraint of still having a reasonable number of spores per strip.

Fig. 6 illustrates the relative behavior of bacterial spores from the biological indicator and the contamination in the load to be sterilized. The solid diagonal lines on the left correspond to the survivor curves for the several species of resistant organisms in the product to be sterilized. The dashed lines are survivor curves for two spore types that may be used as biological indicator organisms. The solid horizontal lines labeled .05 and .99 correspond to survival levels of 0.051 and 4.605 microorganisms respectively. When the initial number of organisms is sufficiently larger, then the probability of one or more organisms surviving is .05 for an average survival level of 0.051 and .99 for a survival level of 4.605.

The design sterilization cycle as illustrated in Fig. 6 is 15 minutes at 121°C; we have decided that the biological indicator should cross the .05 probability line at this time condition. The biological indicator N_o population is designed so that the respective survivor curve passed through the point .05 probability and 15 minutes. This requirement yields N_o's of 1.62×10^6 and 50 for D-values of 2.0 and 5.0 respectively. Given these survivor curves for D = 2 minutes and D = 5 minutes, it is observed that at approximately 11 and 5 minutes respectively the average number of microorganisms is 4.650, i.e., the probability of a positive biological indicator at these times is about 0.99.

In the example it is indicated that 3 minutes are required to reduce the microbial population in the load to be sterilized to a level of 10^{-5}. By using either of the above biological indicator organisms at these specified N_o levels, we observe that the probability of not detecting a heating time below 3 minutes at 121°C is very small.
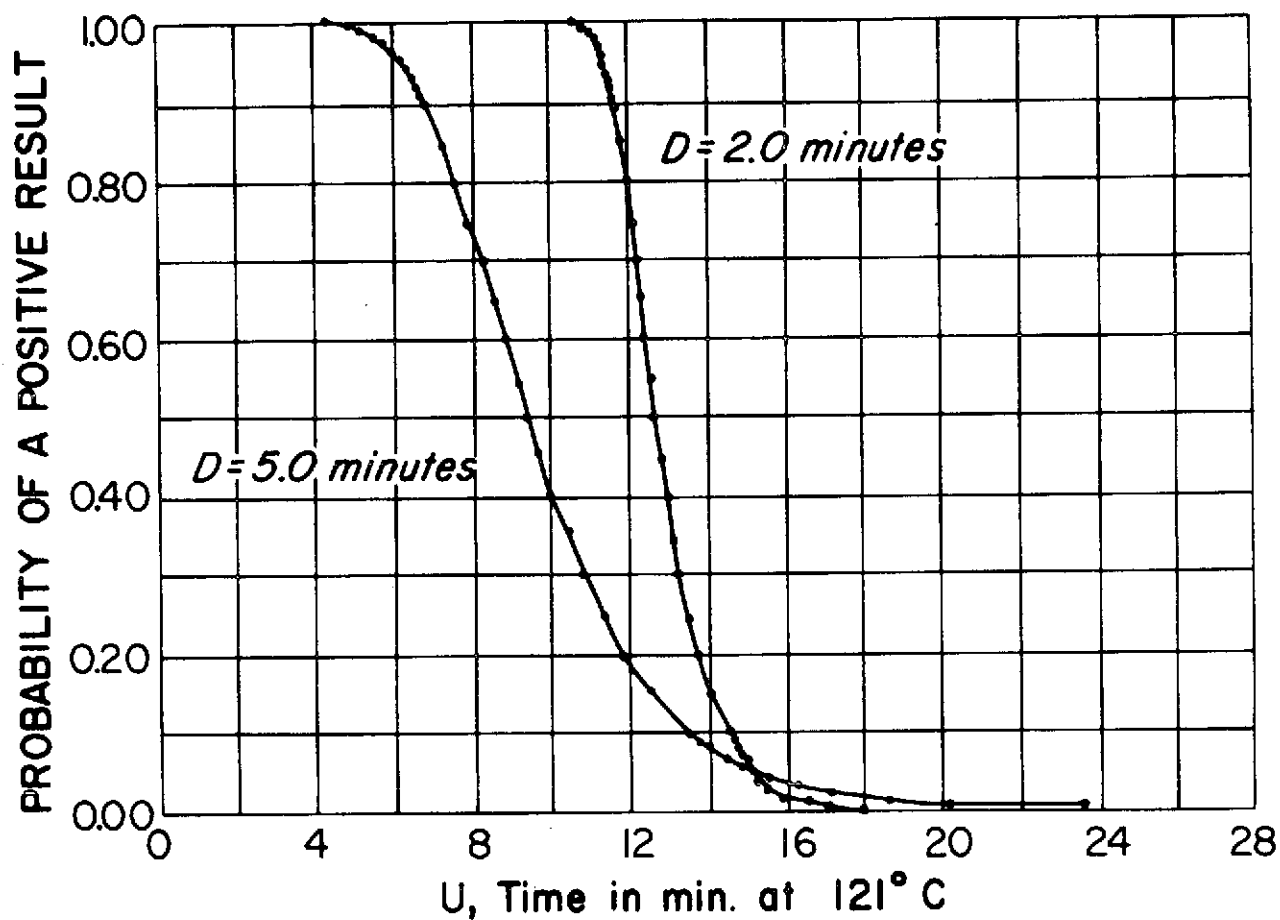
Figure 5: A graph of the probability of a positive result as a function of heating time U for spores having $D_{121°C}$-values of 2.0 minutes and 5.0 minutes based on a sterilization cycle design of fifteen minutes at 121°C.
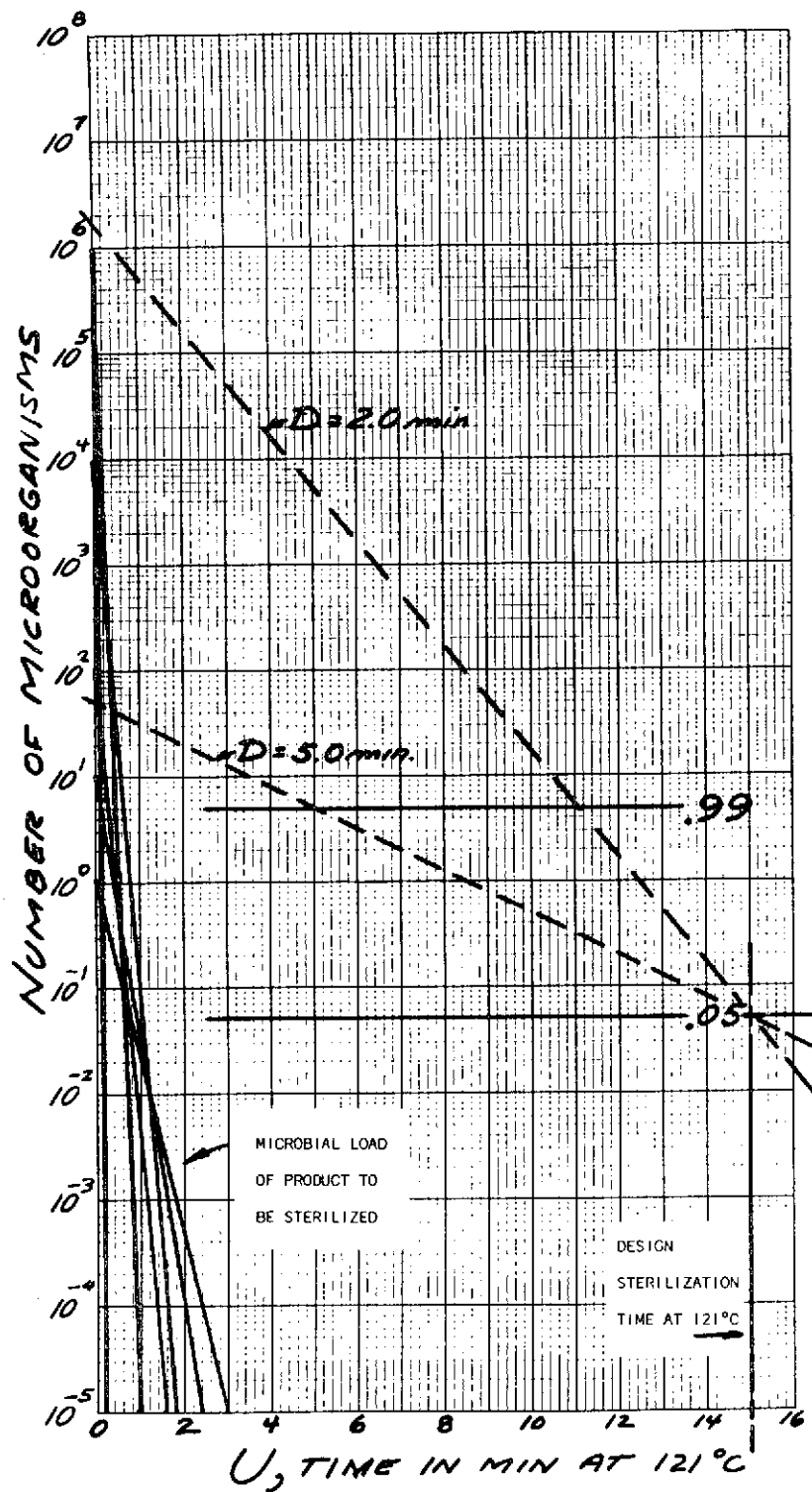
Figure 6: A graph showing the characteristics of a biological indicator system when monitoring the sterilization of a product with a typical microbial load.

Producing biological indicators

The several main steps in producing a biological indicator are listed in Table 2. In general, growing, harvesting, and cleaning the spores will utilize those methods accepted in the literature for the particular spores that are being used.

Table 2

Steps in Producing a Biological Indicator

1. Grow spores.
2. Clean and concentrate spores.
3. Calibrate--develop a thermal destruction rate curve.
4. Design biological indicator.
5. Manufacture spore strips.
6. Test biological indicator to see that it meets specification.

At this point in time, we do not completely understand the relationship of nutrients in the growth medium and the heat resistance of the spore crop. Therefore, the production of spores is still empirical. Seemingly negligible variation in the growing procedure and in the substrate can produce significant changes in the resistance of the spore crop. In our opinion, for the production of spores for biological indicators, the ideal growth substrate should produce a spore crop of average resistance for that species and should have the characteristic that a small change in the growth substrate will not significantly alter the heat resistance of the spores.

In the selection and use of growth and recovery media for spores, it is important to keep in mind that spores are sufficiently sensitive to external conditions that we can use them as biological indicators to monitor a heat sterilization process. Certainly if these spores are able to measure small quantities of heat, they are able to monitor the nutrient conditions of the media on which they are grown. One measurement of a change in the nutrient condition of the growth media will be a change in the heat resistance of the spores.

One of the important processes in the overall scheme of producing biological indicators is the calibration of the newly grown, cleaned, and concentrated spores. By calibration, we mean the establishment of a thermal

destruction curve for the particular lot of spores using the same suspension of deposition method that will be used in the final biological indicator. Recovery will be carried out using the same growth media that will be used in the final growth-tube evaluation.

Each of the spores must be calibrated before they can be used in spore strip manufacture. In the calibration tests, ideally the number of spores per calibration test spore strip should be approximately the same as the number of spores on the final manufactured spore strips. It will not always be possible to achieve this ideal; however, the number of spores on the calibration-test strips should not differ from the number on the final spore strip by more than a factor of ten. Sufficient data in the form of numbers of survivors as a function of heating time should be generated to establish the survivor curve through the zone of use. The shape of the survivor curve is in itself a quality control attribute; the reproducibility of spore production methods can be measured by comparing the shapes of the survivor curve of the different lots of spores.

The calibration of the initial spore suspension and the quality control checks of the biological indicator system should all be conducted so that the spores are exposed in the same manner as in the final sterilization device. Pflug and Schmidt (1968) described the methods that are commonly used for determining thermal resistance. When the spore strip is in direct contact with the steam in the autoclave, it is our opinion that the method developed by Pflug and Augustine (1962) using small cups in a tray heated in a miniature retort, has considerable merit (see Figs. 7 and 8). The miniature retort system (Schmidt, Bock and Moberg, 1955) is a relatively inexpensive piece of laboratory apparatus and has the ability of subjecting microorganisms in cups to direct steam with a sufficiently short come-up and cool time so that the lag correction factor can be neglected (see Fig. 9).

Miniature retorts can be used in groups of two to six although our recommendation is a group of two or three. The availability of two or three miniature retorts means that the time involved in making a thermal resistance study is relatively short since two or three samples can be heating simultaneously. The use of the cup-tray system eliminates the process of sealing glass tubes, breaking glass tubes, and the accompanying problems. It has the advantage that the spores are tested in an environment more nearly like the one in which they will be finally used. If reproducible results are to be obtained, exposure times and temperatures in a miniature retort system
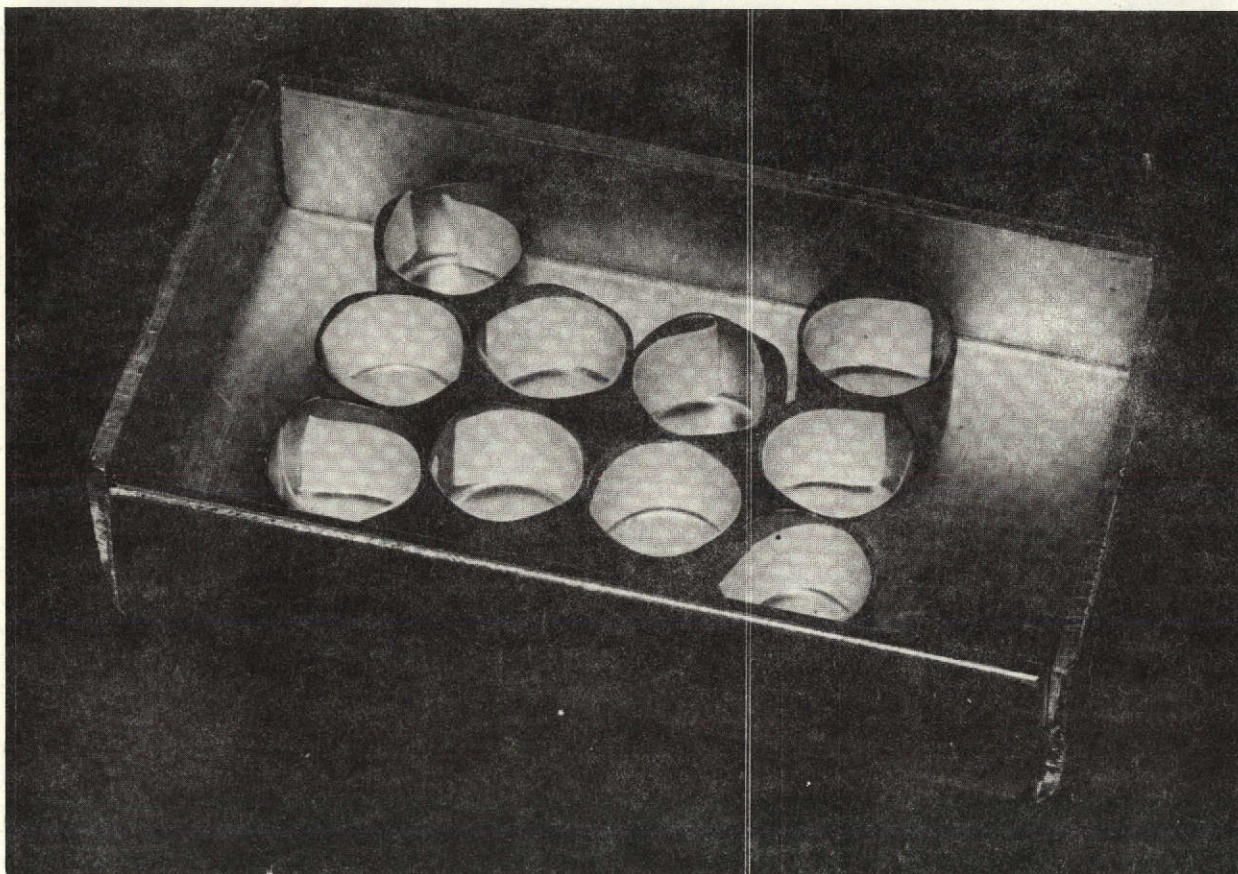
77

Figure 7: An aluminum tray holding ten thermal-death-time cups. The tray and cups will be sterilized in a miniature retort.
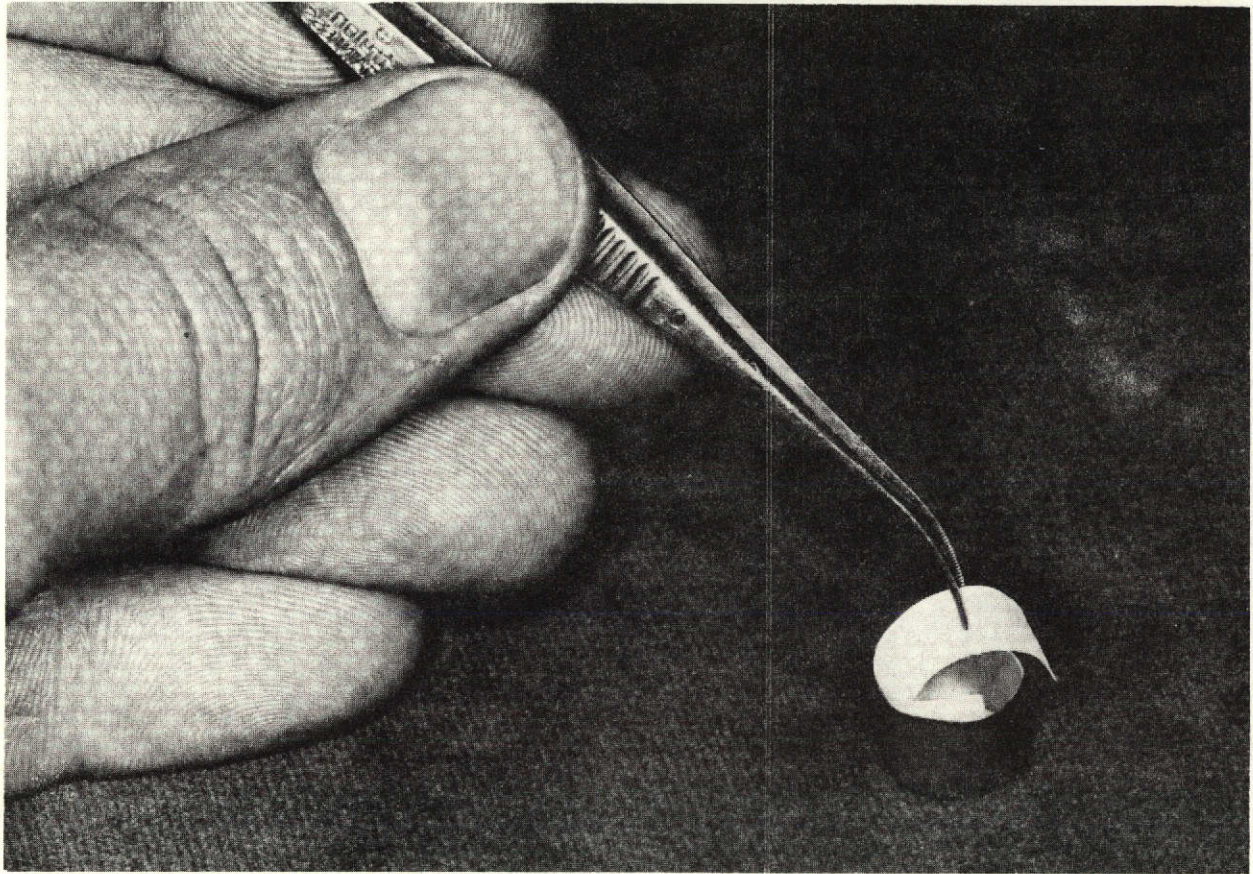
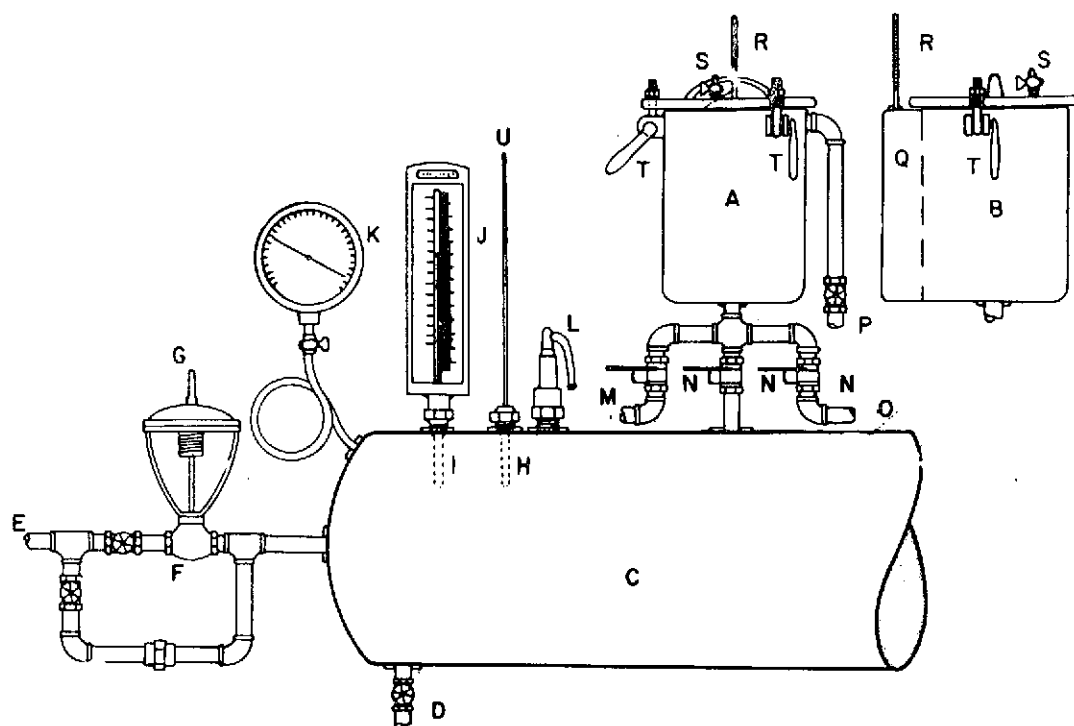Figure 8: A cup with a spore strip inside.

Figure 9: A miniature retort system that is ideally suited
for developing wet heat (saturated steam) thermal resistance
data of bacterial spores.

as in all other thermal resistance systems must be accurately controlled.

The laboratory test method must subject the biological indicator to the same conditions as in the system to be monitored if the results of the test are to be meaningful.

Microbiologists who are analytically inclined will be interested in determining the D-value of the survivor curve and perhaps designing the biological indicator and finally the spore strips analytically. However, it is not necessary to proceed analytically; we can simply establish a line that is parallel to the survivor curve passing through the design point of our sterilization cycle as is shown in Fig. 10. After establishing the value of N_o for the biological indicator, we divide this by n, the number of spore strips per biological indicator. These calculations are all shown on Fig. 10.

The production of spore strips can be done in a great many ways. In the laboratory we simply inoculate filter paper strips with a pipette. Obviously, where a large number of spores must be manufactured, automated methods will be used.

The final biological indicator must be evaluated under simulated conditions of use to be sure that it meets specification.

Biological indicators should be stamped with a time limit for use. Storage conditions should be specified. In our opinion, the design specifications of a reject and accept "time at a temperature" rather than the numbers and/or type of spores should be shown on the label. The performance specification is primary; the number and species of spore may be desirable but are secondary.

The usefulness of any biological indicator will to a large extent depend on the tightness of the manufacturing specification and the degree to which the final product meets these specifications. A statistical quality control program throughout manufacturing and testing is a must for the assurance of the quality and hence usefulness of the product.

The use of biological indicators to monitor heat sterilization cycles

The primary steps involved in using a biological indicator are listed in Table 3. The last step as far as the producer was concerned and the first step as far as the user is concerned appear similar. They represent almost identical steps in the quality control program of two different operations. The producer certainly wants to evaluate his product to see that the product he ships meets specifications. The concerned user conducts quality control tests on the raw material coming into his operation. In essence these two
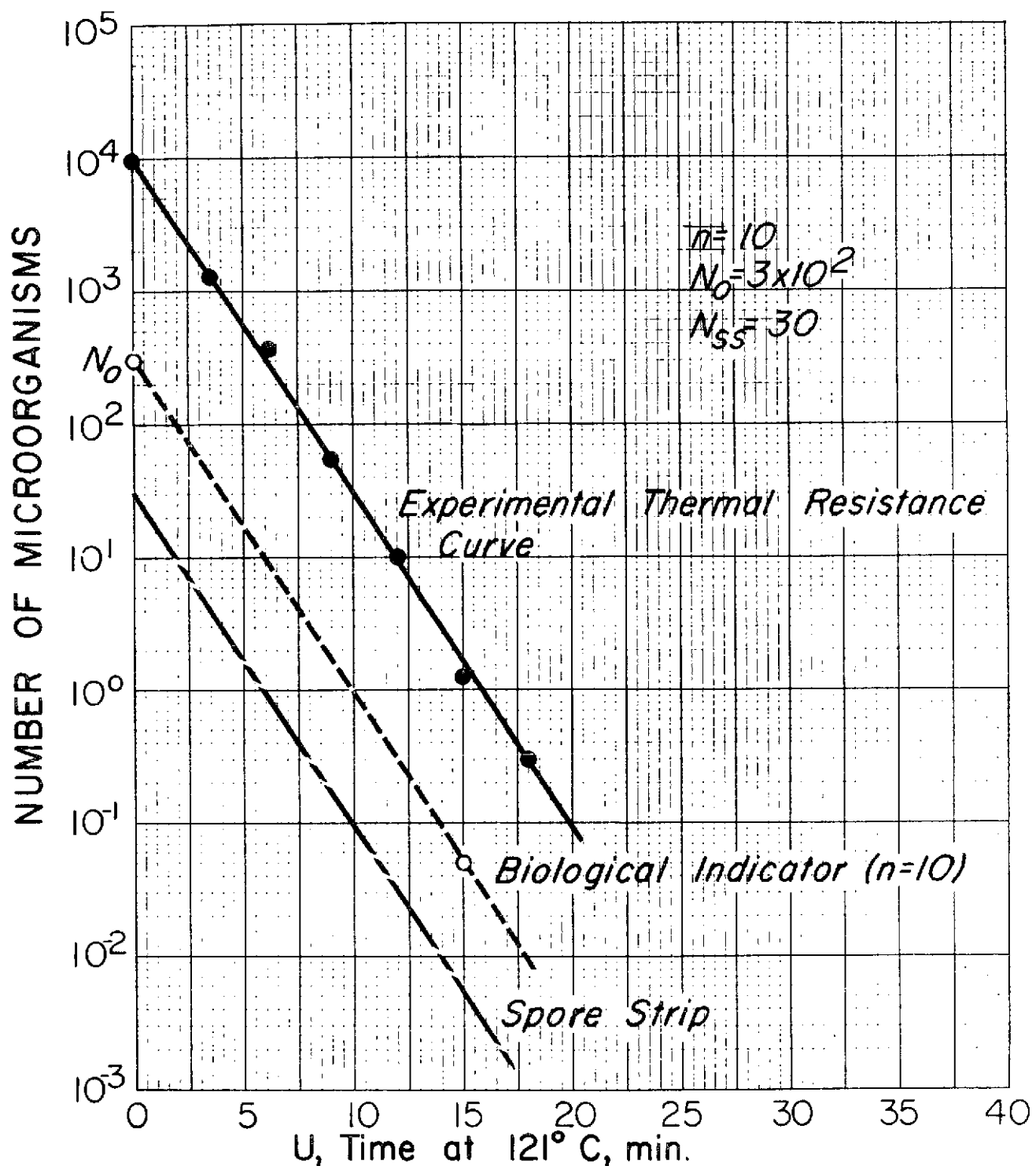
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Figure 10: A graphical method of designing biological indicators: on this graph we show the experimental thermal resistance curve A, the biological indicator curve, (which must go through the design sterilization cycle point, in this case 15 minutes with a probability of 0.05) and finally the spore strip curve C which is N_o for the biological indicator divided by the number of spore strips in the load.

tests are the same; however, they may vary in magnitude in that the producer will certainly check the quality of each lot whereas the user may only check at regular intervals.

Table 3

Steps in Using a Biological Indicator

1. Check biological indicator to see that it meets specifications.
2. Place biological indicator in load to be sterilized.
3. Aseptically transfer exposed spore strip to tubes of growth media.
4. Evaluate tubes for growth.

The placing of the biological indicator in the load of product to be sterilized will probably be based on experience. If a large number of units are sterilized in which each unit heats almost identically to all other units, then the individual spore strips of the biological indicator system, ten in our example, probably would be randomly distributed throughout the load. In very large sterilization systems it may be necessary to have a larger number of spore strips distributed throughout the load in order to have all parts of the load adequately monitored.

If the load of objects being sterilized is small and there is a definite zone of slowest heating then it may be desirable to locate the biological indicators selectively in this zone of slowest heating.

The spore strips must be transferred aseptically from their location in the load of product being sterilized into the tube of growth medium. The laminar flow bench with appropriate controls and aseptic technique has reduced the number of false positives due to outside contamination during this operation; however, it is imperative that good technique be used and maintained. The object containing the spore strip should be protected and handled carefully during transport from the sterilizer load to the laboratory.

Evaluation of the final tubes for growth is made after specified incubation times. It may be desirable to subculture positive tubes to determine whether or not these microorganisms are similar to the spores on the spore strip or whether they are contaminants.

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Summary and conclusions

We have a great deal of confidence in bacterial spores. We have worked with them a long time and our experience has been that they will react in a reproducible fashion if the conditions to which they are subject are constant. In our opinion, bacterial spores are some of the most sensitive transducers we have available today; they can probably measure both chemical and physical conditions that we cannot analytically measure. We believe that much of the variation that is attributed to bacterial spores is not variation in the spores but variation in the system used to evaluate the spores. We believe that there is a terrific opportunity ahead in the use of biological indicators, specifically bacterial spores, for monitoring physical and chemical variables.

In summary we want to re-emphasize some of the characteristics of biological indicators:

1. A biological indicator monitors a sterilization process in terms of the design sterilization cycle. In wet heat the design sterilization cycle is exposure of a product to saturated steam for a prescribed time at a temperature.

2. The information obtained from the use of a biological indicator in a sterilization cycle is directly related to the information going into the design of the biological indicator system. A properly designed biological indicator will indicate with a high degree of certainty, whether the sterilization cycle is below the predetermined minimum, (in which case the lot is to be rejected) or if it is equal to or above the designed sterilization cycle (in which case the load is accepted).

3. The results from the use of biological indicators in a sterilization cycle are a function of the total number of spores in the sterilizer (N_o), the destruction characteristics of the spores under the conditions of exposure (D_T), and the temperature coefficient (z) of the spores on the spore strips.

4. When we use the term biological indicator, we are referring to a sterilization monitoring system that will signal "accept" or "reject."
    A spore strip is a piece of filter paper that has been inoculated with spores. We believe it is appropriate for us to ask, "Are we producing or using a biological indicator or just spore strips?"

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